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Taxonomy, biogeography and ecology of Andean tardigrades at different spatial scales

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Taxonomy, biogeography and ecology of Andean tardigrades at different spatial scales

by

BALBINA P. L. RAMSAY

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of
DOCTOR OF PHILOSOPHY

School of Biological and Marine Sciences
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Abstract

Micrometazoans are animals smaller than 2 mm. Their biogeography is poorly understood, and tardigrades provide a tractable phylum for exploring distribution patterns at a variety of scales. *Polylepis* forest habitat offers considerable advantages for making tardigrade comparisons across a wide range of scales in the Andes. This thesis aims to improve identifications of tardigrades with a character matrix approach, to assess the relative importance of habitat and bryophyte host on tardigrades, to describe the fine-scale spatial structure of tardigrade assemblages, and to estimate the sampling effort required for a reliable estimate of tardigrade diversity within *Polylepis* forest.

Samples of bryophytes and lichens were collected from *Polylepis* forest and neighbouring habitats, and the tardigrades extracted and identified, mostly to operational taxonomic units. Some new species were discovered during the course of this work; one is described here. Abundance, diversity and composition of tardigrade samples were compared quantitatively.

The thesis presents the first example of a character matrix for a tardigrade genus, bringing together information for the genus *Isohypsibius* from many different sources and describing suites of characters for each species. It will facilitate identification within the genus in future.

Tardigrade assemblage data were highly variable within the samples, with empty samples dominating one study. Analysis of one forest site indicated that at least 50 samples would be needed to characterise the tardigrade diversity there. Although both were important, habitat-scale effects were more influential on tardigrade abundance, diversity and composition than host-scale effects. In both cases, microenvironmental and resource filters are the likely mechanisms driving these differences.

Based on the results, recommendations are made for expanding such research into broader geographical scales: standardising sample volume, replicate sampling across hosts on the forest floor, recognising the importance of habitat-scale effects when selecting study sites, and the development of character matrices for tardigrade genera.

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Spatial scale and the biogeography of Tardigrada: *Polylepis* forests in the Andes as a model system

Small organisms that cannot be seen easily by the human eye are abundant, contribute significantly to global biodiversity, and play vital roles in ecosystem function, as part of trophic relationships, energy transfer, and cycling of nutrients and other chemicals (Green *et al.*, 2004, Sohlenius *et al.*, 2004).

Several terms are used interchangeably to refer to organisms of a very small size, such as microorganisms, meiofauna and micrometazoans. Furthermore, there is no consensus about their use, leading to inconsistency and potential confusion (Hughes Martiny *et al.*, 2006).

The term “microorganism” refers to an organism from the Bacteria and Archaea domains, but can also include small organisms of the domain Eukarya, such as unicellular algae, some fungi and protists. Sometimes, microorganisms have been defined by size, e.g., microorganisms with a mass of less than 10 µg and a length of less than 500 µm (Hughes Martiny *et al.*, 2006).

“Meiofauna” have also been defined by their size, as organisms which pass through a sieve mesh of 500–1000 µm but are retained by a sieve of 44–63 µm (McIntyre, 1969, Rundle *et al.*, 2002, Giere, 2009). Meiofauna have been studied in aquatic systems but there are fewer studies in terrestrial ecosystems (McInnes and Pugh, 1998). Some definitions of meiofauna restrict the term to aquatic organisms, so according to this view, organisms of this size in terrestrial situations would not classify as meiofauna.

In this thesis, the term “micrometazoan” is used to refer to organisms less than 2 mm (Guil, 2011), such as tardigrades and other meiofauna like rotifers, nematodes, turbellarians, gastrotrichs and copepods.

The distribution patterns of tardigrades and other micrometazoans in time and space—their biogeography—is poorly understood at present. Despite their abundance and importance, most of these small organisms suffer greatly from what have been termed the Linnean and Wallacean shortfalls (Lomolino *et al.*, 2006): most species have yet to be discovered and described, and their distribution patterns are unknown. (Green *et al.*,

2004, Fierer and Jackson, 2006b, Green and Bohannan, 2006) Nevertheless, tardigrades, for example, have been found throughout terrestrial ecosystems from Antarctica to thermal springs, from beaches to mountaintops, and from simple rock surfaces to complex tropical forests (Marcus, 1928, Kathman and Cross, 1991).

Although some species of macroscopic organism are cosmopolitan (found across large parts of the world), the majority show some degree of endemism reflecting evolutionary history and dispersal limitations, restricted to particular biogeographical provinces, regions or even specific sites (Lomolino *et al.*, 2006). However, it cannot be assumed that the biogeographical patterns widely described for macroscopic organisms also apply to much smaller organisms, for a variety of reasons. First of all, smaller organisms are more easily dispersed than larger ones, passively by wind and water (Nelson and McInnes, 2002). Finlay (2002) argued that any organisms less than 1 mm in size would have unlimited dispersal. So, in contrast to the geographically-restricted distributions of macroscopic organisms, the ubiquitous dispersal of free-living microorganisms unlocks almost unlimited geographical ranges (Finlay and Fenchel, 2004).

In addition, abundance is inversely proportional to body size, as a general rule, and so microscopic taxa usually contain great numbers of individuals (Damuth, 1981, Schmid *et al.*, 2000, Finlay, 2002). For example, it has been estimated that the abundance of organisms with a size of 10 μm will be about 12 times greater than that of organisms with a size of 10 cm (Finlay, 2002). Large population size increases the likelihood of dispersal by chance, compared with organisms with smaller populations.

Furthermore, small organisms often have better abilities than macro-organisms to survive long-distance transport (Hughes Martiny *et al.*, 2006), illustrated by the dormant life stages of *Bacillus* (Green and Bohannan, 2006). In turn, large population sizes and effective long-distance dispersal would be expected to reduce the opportunities for allopatric speciation (as a result of geographical isolation), stochastic extinction events and the impact of historical factors like continental drift. (Hall and Raffaelli, 1991, Fenchel and Finlay, 2003, Finlay and Fenchel, 2004, Esteban and Finlay, 2007)

The biogeography of microorganisms has been assumed to follow this paradigm, usually referred to with the expression “everything is everywhere, but the environment

selects”, often attributed to (Bass-Becking, 1934), but based on the original ideas of Martinus Berijerinck (O'Malley, 2007). More recently, this concept has been referred to as the Ubiquity Distribution Model (Finlay and Fenchel, 2004).

The Ubiquity Distribution Model admits the existence of a transition size zone, across which organisms change from being cosmopolitan to having a restricted distribution, suggested at size ranges of 1 mm to 10 mm (Lawton, 1999). But this proposal has been challenged. For example, (Smith and Wilkinson, 2007) found restricted distributions in *Nebela vas Certes* (Protozoa: Amoebozoa: Arcellinida) which measured between 90–210 µm in length. There are currently too few studies to determine whether a transition size exists and, if so, at what sizes the transition occurs.

After a long period of unquestioned acceptance, the Ubiquity Distribution Model has more recently been challenged. Conceptually, some of the assumptions of the model may not always hold true for microorganisms. For example, the idea that microscopic organisms have relatively low species diversity ignores their very short generation times, the long periods of time over which speciation might have occurred, and the readiness with which gene transfer occurs without involving sex (Foissner, 2008). Taken together, these arguments suggest that local speciation might occur readily in microscopic organisms, at rates faster than rates of dispersal around the world can match.

Papke and Ward (2004) have argued that geographic barriers to microbial dispersal are relatively common and physical isolation is an important driver of microbial evolution. They cite a handful of studies as evidence for the occurrence of microbial endemism, including work on hot spring microbes (Papke *et al.*, 2003, Whitaker *et al.*, 2003, Jones *et al.*, 2016) and soil pseudomonads (Cho and Tiedje, 2000).

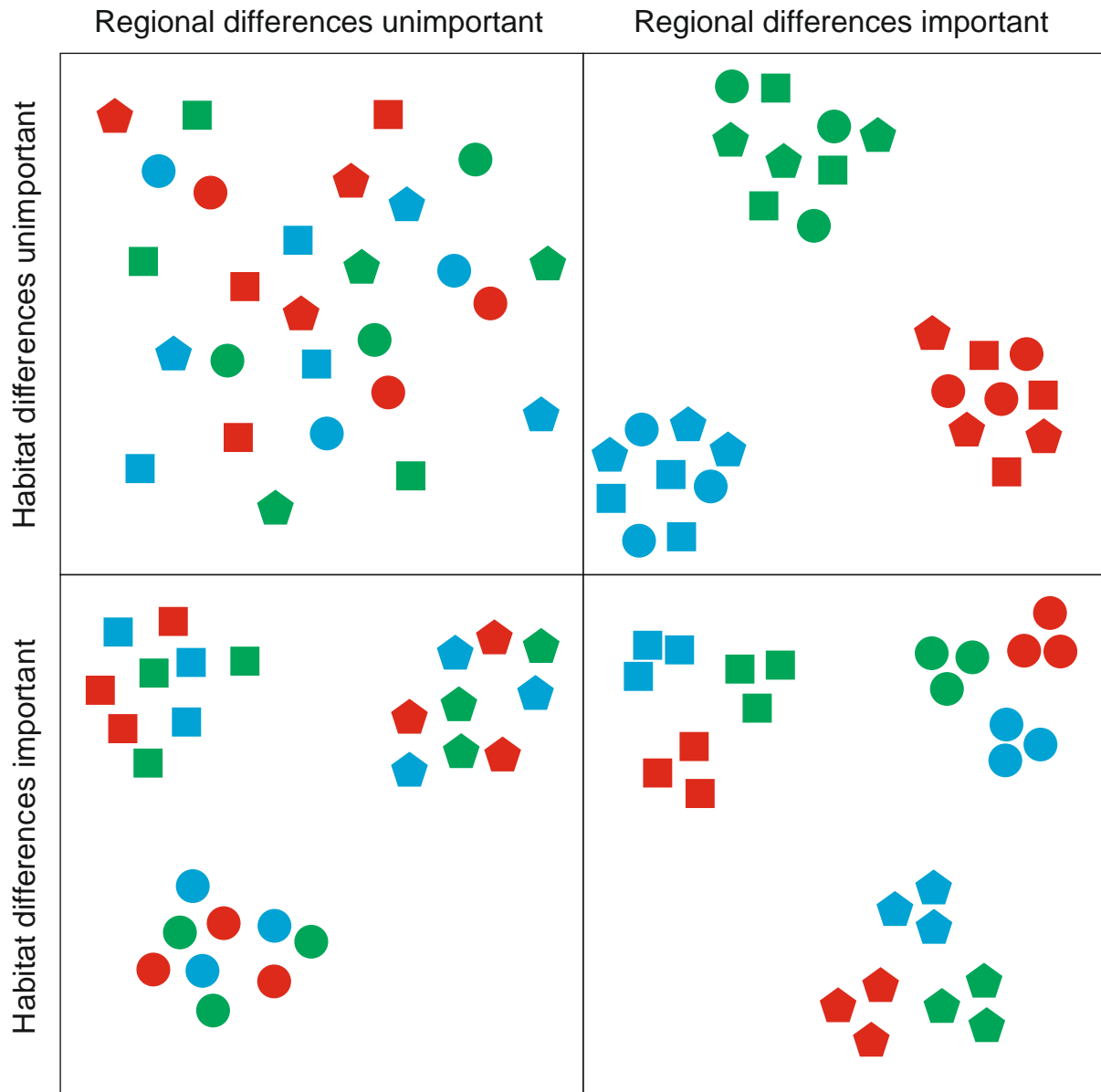
Low sampling effort (Finlay, 2002, Bryant *et al.*, 2008) and the use of inappropriate methods to collect and identify microscopic organisms (Green and Bohannan, 2006) is likely to have resulted in serious underestimation of local biodiversity in these organisms, as well as obscuring differences between regions (Foissner, 2008). Nowadays, the widespread use of molecular approaches, especially with microorganisms, is revealing the great extent of this missed biodiversity (Fierer and Jackson, 2006a, Smith *et al.*, 2008, Czechowski *et al.*, 2012)

As more studies of microscopic-level biodiversity are published, it has become clear that some smaller organisms do indeed have restricted distributions. This has led to the proposal of an alternative concept for the biogeography of microscopic organisms: the Moderate Endemicity Distribution Model, sometimes also referred to as the Biogeography Theory [for Microscopic Organisms] (Finlay, 2002). This paradigm states that some (but not all) microscopic taxa have restricted distributions because of limited dispersal. Local conditions still determine fine-scale patterns according to the individual requirements of particular taxa, as with the Ubiquity Distribution Model.

Fierer *et al.* (2007) and GuilSanchez-Moreno *et al.* (2009) in their studies of bacteria and tardigrades, respectively, demonstrate distribution patterns more like those of larger animals. (Heger *et al.*, 2009) showed support for the Moderate Endemicity Distribution Model with testate amoebae (a good model group for free-living protists), in which some species were small enough to be passively transported over long distances but others were too large for this to happen. This clearly links with earlier comments on the size at which a cosmopolitan distribution might transition to a restricted one.

At the centre of this debate is the complication of scale. Often, biogeographical patterns have been summarised in coarse-scale maps showing global or regional distribution patterns, but this masks other patterns at finer scales, usually relating to niche requirements (Lomolino *et al.*, 2006). A classical example of this is Erickson's (1945) study of patchiness in *Clematis fremontii* var. *riehlii* plants at a variety of scales. But such patchiness at fine scales has also been clearly demonstrated for some microscopic organisms (Kassen and Rainey, 2004). The complication of scales is, therefore, that occupancy and abundance varies considerably at small spatial scales and generates high community dissimilarity over relatively small distances (Declerck *et al.*, 2011), and it is important to resolve these fine-scale issues when investigating patterns at coarser-scales. There is no single correct scale on which to describe biodiversity patterns (Greig-Smith, 1964, Steele, 1989), but if the scale of description is changed, the behaviour of the pattern can move from unpredictable, unrepeatable individual cases to collections of cases with regular patterns for which generalizations can be made (Levin, 1992).

Figure 1.1. A conceptual summary of the compositional outcomes of two competing theories of micrometazoan biogeography. Regional biogeographical differences might be important (left-hand panels) or unimportant (right-hand panels). Habitat differences might be unimportant (upper panels) or important (lower panels). Samples are represented by the symbols, the colour of which denotes regions, and the shape of which denote habitats. Similarity in metazoan composition is represented by closeness in the diagram. For example, in the lower right panel, samples from each habitat are clustered together because they are more similar to each other than to those of other habitats, and samples from regions are clustered together within the habitats. Adapted from an original figure by (Hughes Martiny *et al.*, 2006).



Thus, ecological processes and patterns are scale dependent, an important task is to identify distinctive spatial scales at which species react most strongly (Schooley, 2006). These characteristics scales of response may differ between species and may be linked to mobility and other history features, and can ultimately lead to significant

understanding of the driving mechanisms (Steele, 1989, Wiens, 1989, Levin, 1992, Schooley, 2006). To illustrate, Fig. 1.1 compares four scenarios representing the combination of outcomes for a group of microscopic organisms, depending on whether or not coarser-scale, regional patterns and/or finer-scale habitat patterns are important. Clear, statistically testable outcomes can be seen when comparing the impacts of these two scales, with direct relevance to the models described earlier.

One of the problems in biogeographical studies of microscopic organisms is that coarse-scale patterns are being considered before fine-scale patterns have been properly determined. A knowledge of distribution patterns across a range of scales will not only increase our understanding of microscopic biodiversity, also provides a better understanding of the spatial scaling rules that govern the organisms, but more studies are needed to progress (Green and Bohannan, 2006, Bryant *et al.*, 2008). Generally speaking, the distribution patterns at large scales of the microscopic organisms have not been well-studied because of the technical difficulties in carrying out such studies, concerning sampling effort, processing time, and taxonomy (Green *et al.*, 2004).

The micrometazoans (including the meiofauna) comprise an interesting taxonomic group for biogeographical study. They represent a size range which crosses the likely transition zone between the more cosmopolitan taxa and those with potentially restricted distributions. They have been studied to some extent in aquatic systems (e.g., McIntyre, 1969, Rundle *et al.*, 2002), but there are fewer studies in terrestrial ecosystems (e.g., McInnes and Pugh, 1998). Furthermore, although studies have been carried out in the Northern Hemisphere they are very rarely done in the Southern Hemisphere (Esteban and Finlay, 2007).

Within the micrometazoans, some groups of the meiofauna have been relatively well studied at local spatial and temporal scales (Commito and Tita, 2002). Despite these contributions to the understanding of local dispersal, most aspects about the distribution of meiofauna remain poorly understood (Azovsky *et al.*, 2004). For example, the link between marine foraminifera and environmental conditions has been documented at fine scales (Alve, 1999), as has the vertical distribution pattern of marine nematodes, linked to food supply (Adao *et al.*, 2009), but coarse-scale descriptions of distribution patterns are missing for both groups (Traunspurger, 2002). Virtually nothing is known about modes or degree of dispersal of microturbellaria

(Kolasa, 2002). Such undersampling creates precisely the difficulties discussed earlier when attempting to describe distribution patterns (Green and Bohannan, 2006).

Among the micrometazoans, tardigrades present a useful model group for study. They are an important group of micrometazoans, which form part of the terrestrial and freshwater marine meiofauna (Ramazzotti and Maucci, 1983, Bertolani *et al.*, 2009).

Tardigrades are hydrophilic microscopic invertebrates belonging to the phylum Tardigrada and are more commonly known as 'water bears'. They were first discovered in 1773 by Goeze (Romano, 2003). The phylum is composed of animals with a body size range between 50–1200 μm (Dewel *et al.*, 1993). They are complex organisms with five body segments, four pairs of legs ending in claws, complex mouth and pharynx systems, and no respiratory or circulatory systems (Kinchin, 1994). Reproduction is either sexual or by parthenogenesis, with males generally smaller in size than females. Tardigrade life span is estimated at 3–6 months. Some species with the ability to enter a period of latency, known as cryptobiosis, which can greatly increase the life span (Ramazzotti and Maucci, 1983). Throughout life, tardigrades undergo various periods of moulting which last from 5–10 days (Walz, 1982).

Tardigrades are grouped into three classes: the Heterotardigrada contains mainly marine and armoured terrestrial tardigrades; the Eutardigrada includes unarmoured freshwater and other terrestrial species; and the dubious Mesotardigrada has been based on a single report of the species *Thermozodium esakii* Rahm with no surviving type specimens from a hot spring in Japan (Rahm, 1937).

Tardigrades are good models to represent micrometazoans in distribution studies. They are relatively abundant in a wide range of situations: from the equator to the poles, in both aquatic and terrestrial environments (Marcus, 1928, Horikawa and Higashi, 2004, Czechowski *et al.*, 2012). They are convenient to work with because they are easy to collect and store, and, since tardigrades can enter dormancy, they are able to survive environmental extremes such as desiccation, significant temperature variations and other extreme conditions (Jönsson *et al.*, 2005, Horikawa *et al.*, 2006b, HorikawaKuneida *et al.*, 2008, Jönsson *et al.*, 2008). This has the additional benefit of providing more time for sample processing. Although processing time is lengthy (associated with sorting and mounting every individual animal), their taxonomy is

relatively well documented compared with other micrometazoan groups. Updated checklists of current taxa are regularly published (e.g., Degma *et al.*, 2017).

At present, information about tardigrade distribution patterns comes mostly from information linked to species descriptions. At all spatial scales, this results in gaps in the understanding of tardigrade abundance and diversity. Some species are known from many different parts of the world, while others are known from just one locality (McInnes *et al.*, 2001). It is not clear whether this represents a true reflection of these distributions or merely results from insufficient collected material—in almost every case, the latter is most likely. A few studies have attempted to examine finer-scale distribution patterns of tardigrades (GuilSanchez-Moreno *et al.*, 2009), but with so little information, it is not clear how representative they are. One feature of sampling tardigrades is their potentially high variability in abundance at fine scales, which can result in patchy datasets with many, few or no organisms in replicate samples, which requires careful planning of effective sampling strategies (Meyer *et al.*, 2003).

As discussed earlier, biogeographical patterns are best studied across a range of scales and it is vital, therefore, to choose potential study sites carefully. For terrestrial environments, the alpine biome offers an interesting option because it is the only biogeographical unit on land with a global distribution (Körner, 2003). Thus, it represents an ideal focus for studying tardigrade biogeography at a variety of scales.

In many parts of the world, alpine environments are heavily fragmented, but the Andes of South America provide a continuous mountain chain from close to the Caribbean coast in Colombia and Venezuela, across the equator in Ecuador, through Peru and Bolivia into Chile and Argentina in the south. This provides potential study areas from 12°N to 55°S. In addition, there are potential biogeographical connections to the Rocky Mountains in North America and to the Antarctic Peninsula to the south.

At a regional level, there are partially isolated massifs or even single volcanic mountaintops separated by warmer land, with clear biogeographical patterns evident in larger animals, *e.g.*, carabid beetles (Moret, 2005). At landscape scales, there are several extensive land cover types over a range of climatic conditions (*e.g.*, grasslands, forests and wetlands at different altitudes). Within these landscape elements, there is structural habitat diversity (*e.g.*, soil, rocks, tree trunks, tree branches in the canopy) and microhabitats (*e.g.*, different host bryophytes in moss fields). Clearly, this presents a

useful range of scales across which biogeographical patterns and their drivers can be studied, and is summarised in Fig. 1.3.

However, at present, there have been few studies of tardigrades in the Andes and their distribution patterns are very poorly known, though new species have occasionally been described from Andean samples, *e.g.*, *Platicrista ramsayi* (Marley 2006), *Isohypsibius condorcanquii* (Kaczmarek et al 2014) *Echiniscus ollantaytamboensis* (Nickel et al 2000).

Woodlands dominated by trees of the genus *Polylepis* occur throughout much of the Andean range, from 10°N to 32°S (Venezuela and Colombia to Chile and Argentina). These woodlands promote biodiversity, offering sheltered richly-structured habitats for a variety of plants and animals, and might have acted as refugia during past climate change episodes (Zutta and Rundel, 2017). *Polylepis* forests are largely monospecific in tree composition. They offer suitable conditions for tardigrades on substrates often densely carpeted with bryophytes and lichens. Although there are 15–33 different species of *Polylepis* depending on author (Kessler and Schmidt-Lebuhn, 2006), they tend to create very similar forest habitats and almost always of a single species. In the lower parts of their elevational ranges, they become mixed with other tree species associated with Andean cloud forest. Thus, these woodlands present an ideal model habitat in which to study biogeographical patterns of tardigrades: they are relatively simple in tree composition, with similar forest structure, across a distance of approximately 5500 km.

For these reasons, the tardigrades of *Polylepis* woodlands form the focus of a long-term project—of which this thesis is a part. The role of environmental filtering of taxa from species pools is crucial in shaping community structure (Kraft *et al.*, 2015), and the project aims to sample tardigrades across a range of scales to determine where key drivers operate to control tardigrade distribution patterns (illustrated in Figs 1.2 and 1.3). At coarse scales, dispersal filters might operate—for example, creating differences between communities in Argentina and Ecuador—while abiotic and biotic environmental filters (*e.g.*, light, temperature, humidity) would more likely dominate at the finer scales of habitat and below. The final filter depicts the importance of positive and negative biotic interactions (*e.g.*, facilitation, predation, competition).

Figure 1.2. Different biogeographical scales referred to in the thesis. A. Continental or Andean scale, from Venezuela to Argentina. B. Regional scale comparing distinct páramo areas, isolated by biogeographical barriers (here illustrated by Ecuador). C. Landscape scale within a distinct páramo, including different altitudes (in this case, El Ángel-Chiles on the Ecuador-Colombia border). D. Habitat scale, comparing neighbouring habitats in a particular location. E. Habitat micro-environmental scale, illustrated here by forest floor, trunk and canopy micro-environments. F. Host micro-environmental scale, considering the fine-scale conditions offered by distinct bryophytes. G. Fine-scale spatial variation, within a particular host and habitat position (here forest floor with a mixed substrate of *Pleurozium schreberi* and *Thuidium delicatulum*).

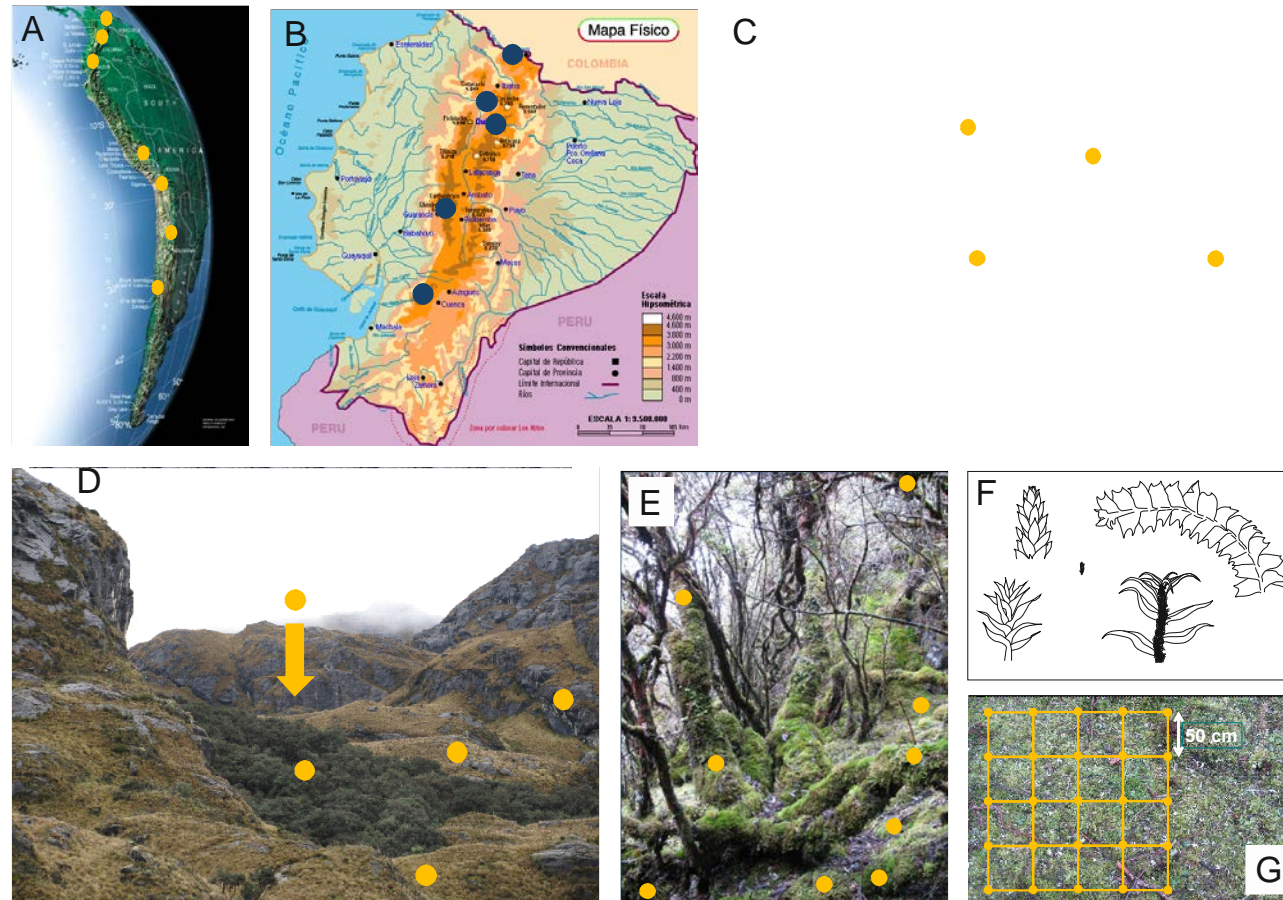
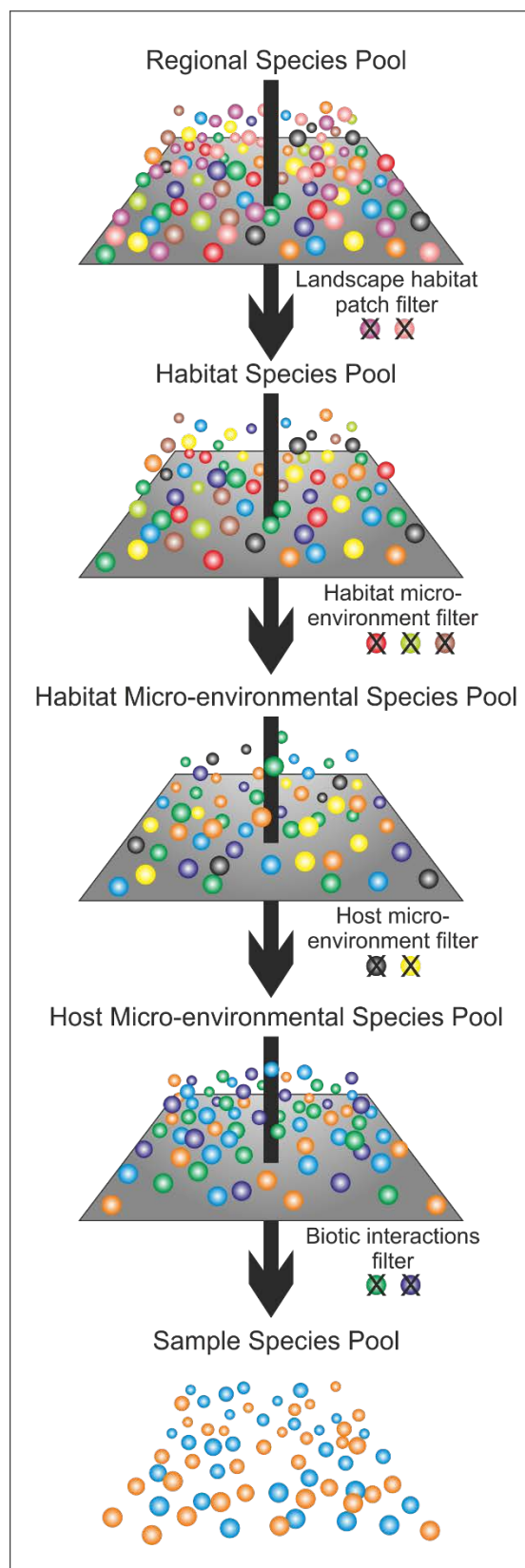


Figure 1.3. Conceptual representation of environmental filtering at different scales for bryophyte-inhabiting tardigrades of Andean *Polylepis* forests. Coloured balls represent different species of tardigrade and each filter excludes certain species.



In order to examine coarse scale biogeographical patterns of tardigrades within *Polylepis* forests, it is vital to understand first the finer-scale patterning of tardigrade communities within *Polylepis* forests, and their context within landscapes. Therefore, this thesis develops some taxonomic strategies to advance the limited resolution of this animal group in South America, and establishes some principles for fine-scale distribution patterns of tardigrades.

Aims of the thesis

The research presented in this thesis focuses on the taxonomy and fine-scale distribution patterns of tardigrades in *Polylepis* woodlands in the Andes. In particular, the aims are to:

1. Develop a character matrix approach to the naming and description of species within a major genus of the Tardigrada.
2. Investigate the presence of tardigrades in a *Polylepis* forest as a pilot study to guide the development of a suitable sampling strategy for later studies.
3. Consider the relative importance of habitat at the landscape scale and bryophyte host within habitats on tardigrade distribution patterns.
4. Explore fine-scale variation in tardigrade assemblages within an Andean *Polylepis* forest, to determine the spatial structure of tardigrade assemblages at this scale and to estimate the number of samples required to obtain a reliable picture of tardigrade diversity.
5. Recommend, based on the work presented, suitable strategies for investigating coarse-scale biogeographical patterns in the Tardigrada, with particular emphasis on *Polylepis* woodlands in the Andes.

Outline of the thesis

Besides the current chapter, the thesis has five more chapters. Four of them present novel research, and the final chapter develops a synthesis and discusses future research. In brief, the basic outline is as follows:

- **Chapter 2. Development of a character matrix to describe a new species of Tardigrada, *Isohypsibius saulrodgersi* sp. nov. (Eutardigrada, Isohypsibiidae), from Ecuador**

The discovery of a new species, *Isohypsibius saulrodgersi* sp. nov., from a *Polylepis* woodland in Ecuador provided a rationale for the development of a character matrix for the genus *Isohypsibius*. The aims of this study were to develop a character matrix for the genus *Isohypsibius*, the third most species rich genus of the phylum, based on all of the published descriptions of the species within it. Such character matrices can facilitate the recognition of species and assist in the description of appropriate characters for new species. This work provides the first complete character matrix for a tardigrade genus. It represents the kind of taxonomic work that needs to be done to support the ecological studies described in later chapters.

- **Chapter 3. The effect of microhabitat on the distribution of tardigrades at high-altitude forest in the Peruvian Andes**

This chapter describes, for the first time, the tardigrade fauna of a high-altitude *Polylepis* forest and adjacent puna grassland in the Peruvian Andes. The influence of three different factors on tardigrade composition were explored: position within the forest and grassland structure (forest floor, tree trunks, canopy branches, grassland), the type of host (bryophytes, lichens, bark), and the substrate (rock, soil, tree). This study was considered a pilot study, and informed the sampling strategy used in later chapters of this thesis.

- **Chapter 4. The structure of tardigrade communities at the landscape scale: the influence of habitat and host**

This chapter compared tardigrade abundance, diversity and composition in bryophyte hosts in three different habitats (bog, forest and grassland) in an Andean mountain landscape. The relative importance of these two micro-environmental filters on tardigrade distributions, as well as their potential interaction, must be understood before comparing tardigrades at coarser scales. On the basis of the findings in this study, recommendations are provided for such coarse-scale comparisons of distribution patterns in tardigrades.

- **Chapter 5. The structure of tardigrade communities at fine spatial scales in an Andean *Polylepis* forest**

This study investigates the fine scale variation in tardigrade assemblages in an Andean *Polylepis* forest. It explores whether bryophyte hosts differ consistently in the species of tardigrade they support, whether there is spatial structure to

tardigrade assemblages on the forest floor, and considers the number of samples required to obtain a confident representation picture of tardigrade diversity at the habitat scale. Once again, recommendations are provided for sampling strategies in coarse-scale comparisons of tardigrade distribution.

- **Chapter 6. Overall discussion**

This chapter briefly synthesizes the results of the thesis and discusses future priorities for research into biogeographical patterns of tardigrades at a range of scales.

2

Development of a character matrix to describe a new species of Tardigrada, *Isohypsibius saulrodgersi* sp. nov. (Eutardigrada, Isohypsibiidae), from Ecuador

Introduction

Traditionally, dichotomous keys have been used by knowledgeable researchers to identify both described taxa and to identify potentially newly discovered taxa. However, if one is not an expert with the specific taxonomic group, using these dichotomous keys can be problematic, requiring educated guesses if a character is not visible for a particular specimen. An alternative solution is the use of a character matrix, which can help in the identification of taxa from species-rich genera, with limited or no access to type material, using older taxonomic descriptions with incomplete details.

Tardigrades are complex microscopic animals, which form part of terrestrial, freshwater and marine meiofauna (Ramazzotti & Maucci 1983; Bertolani *et al.* 2009). Tardigrade taxa are mostly herbivores but also some predators and play a variety of functional roles within meiofaunal communities, (Sutcliffe *et al.* 2000; Sanchez-Moreno *et al.* 2008; Guil & Sanchez-Moreno 2013). A limited number of new taxa are described each year, because there is a limited number of specialist researchers working with this taxonomic group (Kathman & Cross 1991). However, non-specialists who find tardigrades are often interested in identifying the animals but are hindered by the difficulties of working with species descriptions scattered across the literature.

Recent studies have used a combination of morphological and molecular evidence (Kiehl *et al.* 2007; Sands *et al.* 2008) to support the establishment of superfamily rank taxa (Marley *et al.*, 2011), with more recent studies refining the positions of genus-rank taxa (Bertolani *et al.* 2014). However, these papers are written for those with more specialist-level knowledge of the phylum's species rather than the non-expert or lay-researcher.

Isohypsibius Thulin, 1928 is a large terrestrial and freshwater genus with a worldwide distribution, and can be found in diverse habitats and in all climatic zones, (McInnes, 1994). Type material for most of the 134 currently described *Isohypsibius* taxa is not

available for review, which further adds to the difficulty of identifying specimens. There are currently no up-to-date dichotomous keys available to help researchers identify specimens, especially from the Neotropics, without referring back to Ramazzotti and Maucci (1983) plus a very large number of more recent publications, which can be difficult to access.

The discovery of a new species, *Isohypsibius saulrogersi* sp. nov. from a *Polylepis* woodland in Ecuador provided an opportunity to develop a character matrix for the genus *Isohypsibius*. The aims of this study are to develop a character matrix and to use this to assist with differential diagnoses, the description of a new species and demonstrate the usefulness of a character matrix for describing new taxa.

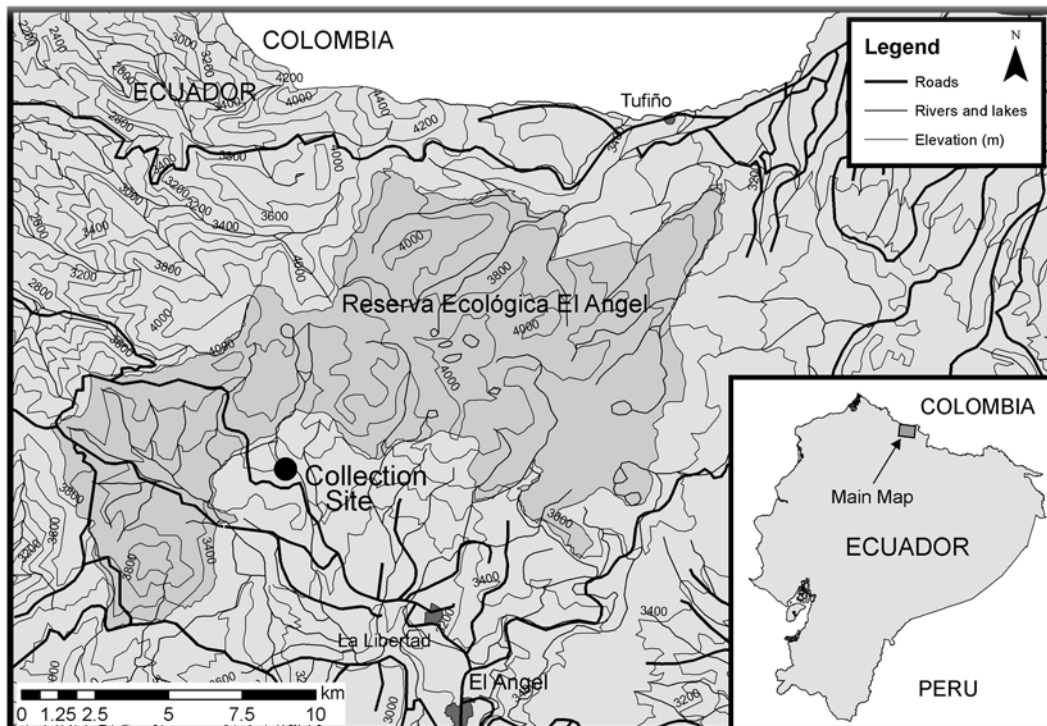
Materials and methods

Bryophyte samples were collected from the ground in a *Polylepis* woodland on the boundary of El Ángel Ecological Reserve, at 3,575 m above sea level (asl) in northern Ecuador in August 2011 (Fig. 2.1). I recorded the location with a Garmin GPSMap 60CSx (Garmin International Inc., Kansas, USA). Specimens of the new species were found in samples containing the following bryophytes: *Pleurozium schreberi* (Brid.) Mitt., *Thuidium delicatulum* (Hedw.) Schimp., *Leptodontium longicaule* Mitt., *Zygodon nivalis* Hampe, and *Chiloscyphus latifolius* (Nees) J.J. Engel & R.M. Schust. (synonym *Lophocolea bidentata* (L.) Dumort.).

Each sample of bryophyte was placed into a manila envelope and allowed to air dry. In the laboratory, each sample was weighed, and rehydrated in a sealed container with tap water for 24 hours. The sample was shaken and rinsed in water and the extract was filtered using two stacked sieves of a decreasing mesh diameter (500 µm and 38 µm). The retained contents on the smaller sieve were washed into a petri dish for examination under a stereoscopic microscope using dark field illumination at x45.

All specimens were mounted individually onto microscope slides in Heinz PVA medium. The mounted specimens were examined and imaged using an Olympus BX53 microscope with phase contrast (PhC), differential interference contrast (DIC) and an Olympus SC50 digital camera with Olympus cellSense Standard version 1.13 Software. Images were produced using Zerene Stacker, Zerene Systems LLC, DMap software and

Figure 2.1. Map of the collection site from El Ángel, Ecuador, at 3,575 m elevation.



The cropped and resized in Adobe Firework CS5.1 v11 (Adobe Systems Inc., San Jose, CA, USA).

Basic anatomy of tardigrades and use of anatomical terms was described by Pilato & Binda (2010). Measurements were made in accordance with Pilato (1981) and Pilato & Binda (2010). All the measurements are given in micrometres. The length of the body was measured from the top of the head to the end of the body, excluding the hind legs. Buccal tube length and level of the stylet support insertion point were measured following Pilato (1981). Buccal tube widths were measured as the external and internal diameters at the level of the stylet support insertion point. Macroplacoid length sequence is given according to Kaczmarek *et al.* (2014). Claws were measured according to Pilato *et al.* (1982) and Beasley *et al.* (2008). The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube expressed as a percentage (Pilato 1981). Formulae for the arrangement of gibbosities is in the overall format suggested by Michalczyk & Kaczmarek (2010): [row number: number of gibbosities per row separated with hyphens, forming rows with the number of gibbosities in each], *e.g.*, [VIII: 2-2-4-4-4-4-2-2]. In some cases, ancillary gibbosities are present and the number of these is indicated in each row in parentheses, either before or after the substantive

row number, reflecting its relative position on the animal, *e.g.*, [X: 3-2-6-4-(2)6-(6)6-4-(2)4-(4)6-3-(2)].

Since most type material for *Isohypsibius* species is unavailable, I used information contained in the original published descriptions to develop the character matrix. The characters used for the matrix were eyespot pigmentation, macroplacoid number, microplacoid number, cuticular sculpturing, presence/absence of tubercles, and the arrangement of gibbosities. Each species was also assigned to broader species-level groups based on similarities in the arrangement of gibbosities.

Results

The character matrix for all 134 described species of *Isohypsibius*, along with the characters of our proposed new species, is presented in Table 2.1.

The proposed new species has a non-smooth dorsal cuticle (shared with 89 other described species), no tubercles (122 spp.), gibbosities in ten rows (20 spp.), an even number of gibbosities in all rows (25 spp.), macroplacoid number being either 3 or 2/3 in the original description (3 macroplacoids 56 spp., 2/3 macroplacoids = 5 spp.), no microplacoid (122 spp.), gibbosities in 9 to 11 rows (35 spp.), and gibbosities arranged in only even numbers within rows (25 spp.). However, the combination of all these characters together was unique and consequently I describe here a species new to science, *Isohypsibius saulrogersi* sp. nov.

Taxonomic account of the new species

Phylum: Tardigrada Doyère, 1840

Class: Eutardigrada Marcus, 1927

Order: Parachela Schuster, Nelson, Grigarick and Christenberry, 1980

Superfamily: Isohypsibioidea Sands, McInnes, Marley, Goodall-Copestake, Convey & Linse, 2008

Family: Isohypsibiidae Sands, McInnes, Marley, Goodall-Copestake, Convey & Linse, 2008

Genus: *Isohypsibius* Thulin, 1928

Isohypsibius saulrogersi sp. nov. (Figs. 2.2–2.6)

Description

Detailed measurements for the holotype and across a wider range of paratypes are given in Table 2.2.

Body colourless (Figs. 2.2-2.4). Eyes visible in six out of ten type series specimens (holotype without visible eyes). Dorso-lateral cuticle, including dorsal surface of leg pair IV, covered with a reticular sculpturing composed of irregular shapes and sized, and ten rows of gibbosities (X: 4-6-4-6-6-6-2-4-4-4). Reticulation size 5.9 μm by 7 μm in the mid-dorsal region of the body between leg pairs II and III; 3.2 μm by 3.8 μm in the mid-dorsal region in the anterior body at leg pair I (Fig. 2.2). Reticulation larger on the gibbosities than on cuticle elsewhere on the dorsolateral region. Diameter of reticular mesh slightly increasing from the dorsolateral anterior section to the medium dorsal plane of the body. Ventral cuticle smooth (*i.e.*, without sculpturing).

Mouth antero-ventral. Six peribuccal lobes present. Peribuccal lamellae absent. Oral cavity without anterior or posterior bands of teeth visible using light microscopy. Thick ring around the top of the buccal tube without additional structures. Bucco-pharyngeal apparatus of the *Isohypsibius* type (Pilato & Binda 2010), including a rigid buccal tube without ventral lamina but with a dorsal and ventral apophysis for the insertion of the stylet muscles with a ridge shape and symmetrical with respect to the frontal plane. Caudal processes of both apophyses pointing backwards and sideways. Pharyngeal bulb with apophysis, with three granular-shaped macroplacoids, all without constrictions. Macroplacoid sequence (1=2<3). Microplacoid and septulum absent (Fig. 2.2).

Asymmetrical double claws of the *Isohypsibius* type (Pilato & Binda 2010), arranged with respect to the median plane of the leg, claw branches arranged secondary-primary-secondary-primary (2121).

The secondary branch and the basal section form almost a right angle. External claws I–III and posterior claws IV slightly larger than internal claws I–III and anterior claws IV. External claws with expanded bases. Internal claws without expanded bases. All primary branches with accessory points. Primary branches on leg IV claws with better developed and more visible accessory points. Lunules absent on all claws, but external claws I–III and posterior claws IV with expanded bases. No cuticular bars near the claw bases of any legs. Eggs not found.

Table 2.1. Diagnostic character matrix for described species of the genus *Isohypsibius*, based on a range of morphological characters. The proposed new species of the genus is also included at the end of the table. "A" = absent, "P" = present, "A+P" = found in some individuals but not in others. Number of gibbosities per row: "E" even only, "O" odd only, "M" mixed odd and even numbers in rows, "?" = character state unknown or unclear from the description, "—" character state not applicable, "[]" = inter row position for gibbosities formulae, within which "()" indicate ancillary gibbosities and "~6~" indicates an inconsistency in the original publication. This table continues over five pages.

Taxon	Eyespot Pigmentation	Macro-placoid Number	Micro-placoid Number	Cuticular Sculpturing	Tubercles	Number of Gibbosities Rows	Gibbosities Per Row	Gibbosities Formula	Species Group Assignment
<i>Isohypsibius altai</i> Kaczmarek & Michalczyk 2006	P	2	1	Smooth	A	0	—	—	?
<i>Isohypsibius annulatus annulatus</i> (Murray 1905)	P	2	0	Smooth	P	0	—	—	annulatus
<i>Isohypsibius annulatus minor</i> (Ramazzotti 1945a)	P	2	0	Smooth	P	0	—	—	annulatus
<i>Isohypsibius arbiter</i> Binda 1980	A	3	1	granulated	A	0	—	—	prosostomus
<i>Isohypsibius archangajensis</i> Kaczmarek & Michalczyk 2004	P	2	0	Scalloped	A	0	—	—	undulatus
<i>Isohypsibius arcuatus</i> Bartoš 1934	P	3	0	Smooth	A	20	—	—	undulatus
<i>Isohypsibius asper</i> (Murray 1906)	P	3	0	Smooth	P	0	—	—	annulatus
<i>Isohypsibius austriacus</i> Iharos 1966b	P	2	0	reticulated	A	10	M	[X: 3-2-4-4-4-4-4-4-2]	tuberculatus
<i>Isohypsibius baicalensis</i> Ramazzotti 1966	P	3	0	granulated	A	0	—	—	granulifer
<i>Isohypsibius baldii</i> Ramazzotti 1945b	?	3	0	flat granulated & reticulated	A	0	—	—	granulifer
<i>Isohypsibius baldioides</i> Tumanov 2003a	A	3	0	flat granulated & reticulated	A	0	—	—	granulifer
<i>Isohypsibius barbarae</i> Pilato & Binda 2002	P	2	0	Scallops	P	20	—	—	undulatus
<i>Isohypsibius bartosi</i> Iharos 1966c	P	2	0	granulated	A	10	E	[X: 4-4-4-4-4-4-4-4-2]	tuberculatus
<i>Isohypsibius basalovoi</i> Durante & Maucci 1973	P	3	0	reticulated	A	9	M	[IX: 2-2-4-4-4-2-4-4-3]	tuberculatus
<i>Isohypsibius belliformis</i> Mihelčič 1971b	?	2 or 3	0	granulated	P	6	M	[VI: 5-2-5-5-2-2]	tuberculatus
<i>Isohypsibius bellus</i> Mihelčič 1971b	?	3	0	Polygons	A	8	M	[VIII: 2-2-2-2-4-2-3-4]	tuberculatus
<i>Isohypsibius borkini</i> Tumanov 2003b	A	2	0	?	A	0	—	—	?
<i>Isohypsibius brevispinosus</i> Iharos 1966a	P	3	0	granulated	A	10	E	[X: 6-4-4-6-4-4-6-4-4]	tuberculatus
<i>Isohypsibius brevitubulatus</i> Rho, Chang & Kim 1997	A	3	0	Smooth	A	0	—	—	?
<i>Isohypsibius brulloi</i> Pilato & Pennisi 1976	A	3	0	reticulated	A	0	—	—	?
<i>Isohypsibius bulbifer</i> Mihelčič 1957	P	2	0	granulated	A	10	M	[X: 3-5-5-4-4-4-4-5-3]	tuberculatus
<i>Isohypsibius cameruni</i> Iharos 1969b	P	2	0	granulated	A	8	O	[VIII: 3-5-3-5-3-5-3-3]	tuberculatus
<i>Isohypsibius campbellensis</i> Pilato 1996	P	3	0	reticulated	A	2	—	caudal portion of body with "2 lines of indistinct protuberances"	?

Taxon	Eyespot Pigmentation	Macro-placoid Number	Micro-placoid Number	Cuticular Sculpturing	Tubercles	Number of Gibbosities Rows	Gibbosities Per Row	Gibbosities Formula	Species Group Assignment
<i>Isohypsibius canadensis</i> Murray 1910	P	3	0	Smooth	A	0	—	—	?
<i>Isohypsibius ceciliae</i> Pilato & Binda 1987	P	3	1	reticulated	A	0	—	—	?
<i>Isohypsibius changbaiensis</i> Yang 1999	A	2	0	?*	A	0	—	—	?
<i>Isohypsibius chiarae</i> Maucci 1987	P	2	0	smooth	A	0	—	—	?
<i>Isohypsibius condorcanquii</i> Kaczmarek Cytan, Zawierucha, Diduszko & Michalczyk 2014	A	3	0	reticulated	P	0	—	—	granulifer
<i>Isohypsibius costatus</i> Mihelčič, 1971b	A	2 or 3	0	reticulated	A	12	M	[XII: 2-2-2-4-3-4-4-2-4-4-5-2]	tuberculatus
<i>Isohypsibius coulsoni</i> Kaczmarek Zawierucha, Smykla & Michalczyk 2012	P	3	1	reticulated	A	0	—	—	?
<i>Isohypsibius cyrilli</i> Mihelčič 1951	P	2	0	reticulated	A	8	M	[VIII: 3-2-3-4-3-4-3-3]	tuberculatus
<i>Isohypsibius damxungensis</i> Yang 2007b	P	3	0	?*	A	0	—	—	?
<i>Isohypsibius dastychi</i> Pilato, Bertolani & Binda 1982	P	2	0	faint spots	A	0	—	—	elegans
<i>Isohypsibius deconincki</i> Pilato 1971	A	3	0	smooth	A	1	E	[I: 2]	?
<i>Isohypsibius deflexus</i> Mihelčič 1960	A	2 or 3	0	smooth	A	0	—	—	?
<i>Isohypsibius dudichi</i> Iharos 1964*	P	2	0	reticulated	A	11	E	[XI: 2-2-4-4-6-4-6-4-6~6~2-4]* [XI: 2-2-4-4(2)-6(2)-4(2)-6(2)-4~6(2)~2-4]*	tuberculatus
<i>Isohypsibius durantee</i> Maucci 1978	A	2	0	granulated	A	9	E	[VIII: 2-4-4-4-4-4-4-4-2]	tuberculatus
<i>Isohypsibius effusus</i> Mihelčič 1971a	A	2	0	reticulated	A	11	M	[X: 2-4-5-5-5-4-5-4-5-4-2]	tuberculatus
<i>Isohypsibius elegans</i> Binda & Pilato 1971	P	2	0	reticulated	A	10	M	[X: 3-2-4-4-4-4-4-4-4-1]	elegans
<i>Isohypsibius eplenyensis</i> Iharos 1970	P	2	0	smooth	A	9	M	[IX: 2-4-2-4-2-4-2-4-3]	tuberculatus
<i>Isohypsibius franzi</i> Mihelčič 1951	P	2 or 3	0	smooth	A	9 or 10	E	[X: 6-6-6-6-6-6-4-4-2-4]	tuberculatus
<i>Isohypsibius fuscus</i> Mihelčič 1972	P	2	0	granulated	A	0	—	—	?
<i>Isohypsibius gilvus</i> Biserov 1986	?	2	0	granulated	A	0	—	—	?
<i>Isohypsibius glaber</i> Durante Pasa & Maucci 1979	A	2	0	smooth	A	9	M	[IX: 2-2-3-5-3-5-3-5-3]	tuberculatus
<i>Isohypsibius glazovi</i> Biserov 1999	P	3	1		A	3	E	[III: 2-2-2]	prosostomus
<i>Isohypsibius gracilis</i> Iharos 1966c	P	2	0	granulated	A	10	M	[X: 3-4-4-4-4-4-4-4-4-3]	tuberculatus
<i>Isohypsibius granditintinus</i> Chang & Rho 1996	A	3	0	smooth	A	?		—	?
<i>Isohypsibius granulifer granulifer</i> Thulin 1928	P	2	0	granulated	A	0	—	—	granulifer
<i>Isohypsibius granulifer koreanensis</i> Iharos 1971	P	2	0	granulated	A	0	—	—	granulifer
<i>Isohypsibius gyulai</i> Mihelčič 1971a	A	2	0	granulated	A	12	E	[XII: 2-4-4-4-4-4-4-4-4-2-2-2]	tuberculatus
<i>Isohypsibius hadzii</i> Mihelčič 1938	A	3	0	hexagonal	A	1	E	[I: 2]	tuberculatus
<i>Isohypsibius helenae</i> Iharos 1964	P	3	0	granulated	A	8	E	[VIII: 2-4-2-4-2-4-2-4]	tuberculatus
<i>Isohypsibius hydrogogianus</i> Ito & Tagami 1993	A	3	0	smooth	A	0	—	—	deconicki

Taxon	Eyespot Pigmentation	Macro-placoid Number	Micro-placoid Number	Cuticular Sculpturing	Tubercles	Number of Gibbosities Rows	Gibbosities Per Row	Gibbosities Formula	Species Group Assignment
<i>Isohypsibius hypostomoides</i> Mihelčič 1971b	P	2	0	granulated	A	2	M	[III: 5-3-2]	tuberculatus
<i>Isohypsibius indicus</i> Murray 1907a	?	2	0	granulated	P	24	E	[XXIII: 6-6-4-8-8-4-8-8-8-8-8-8-4-8-8-8-8-6-6-4]	tuberculatus
<i>Isohypsibius irregibilis</i> Biserov 1992	P	3	0	rugose	A	0	—	—	?
<i>Isohypsibius jakieli</i> Dastych 1984a	P	2	0	smooth	A	0	—	—	?
<i>Isohypsibius jingshanensis</i> Yang 2003	P	3	0	?	A	0	—	—	?
<i>Isohypsibius jinhouensis</i> Yang 2007a	P	3	0	?	A	0	—	—	“poorly described”
<i>Isohypsibius josephi</i> Iharos 1964	A+P	2	0	granulated	A	9	M	[IX: 2-4-4-4-4-4-4-2] [X: 2-2-4-4-4-4-4-4-4-4]	tuberculatus
<i>Isohypsibius karenae</i> Zawierucha 2013	A+P	3	0	reticulated	A	?	—	—	?
<i>Isohypsibius kenodontis</i> Kendall-Fite & Nelson 1996	P	3	0	reticulated	A	0	—	—	?
<i>Isohypsibius kotovae</i> Tumanov 2003a	A	3	0	reticulated	A	0	—	—	tuberculatus
<i>Isohypsibius kristenseni</i> Pilato, Catanzaro & Binda 1989	P	3	0	smooth	A	0	—	—	?
<i>Isohypsibius ladogensis</i> Tumanov 2003a	P	3	0	granulated	A	0	—	—	?
<i>Isohypsibius laevis</i> McInnes 1995	P	3	0	smooth, but x100 irregular reticulate	A	0	—	—	annulatus
<i>Isohypsibius latiunguis</i> Iharos 1964	P	2	0	granulated	A	8	M	[VIII: [2]4-4-4-4-4-2-3]	tuberculatus
<i>Isohypsibius leithaicus</i> Iharos 1966b	A	2	0	reticulated	A	10	M	[X: 3-4-3-4-3-4-3-4-3-2]	tuberculatus
<i>Isohypsibius liae</i> Li & Wang 2006	A	3	0	reticulated	A	12	—	—	undulatus
<i>Isohypsibius lineatus</i> Mihelčič 1969	P	3	0	wrinkled	A	0	—	—	?
<i>Isohypsibius longiunguis</i> Pilato 1974	P	2	0	reticulated	A	10	M	[X: 3-2-4-4-4-4-4-4-1]	elegans
<i>Isohypsibius lunulatus</i> Iharos 1966a	P	2	0	granulated	A	10	M	[X: 3-2-3-4-3-4-3-4-2-3]	tuberculatus
<i>Isohypsibius macrodactylus</i> Maucci 1978	P	3	1	smooth	A	0	—	—	?
<i>Isohypsibius malawiensis</i> Jørgensen 2001	A	3	0	smooth	A	0	—	—	?
<i>Isohypsibius mammillosus</i> Iharos 1964	P	3	0	granulated	A	11	E	[XI: 4-4-4-4-4-4-4-4-4-2]	tuberculatus
<i>Isohypsibius marcellinoi</i> Binda & Pilato 1971	P	2	0	smooth	A	0	—	—	?
<i>Isohypsibius marii</i> R. Bertolani 1982	P	3	0	reticulated	A	0	—	—	granulifer
<i>Isohypsibius mihelcici</i> Iharos 1964	P	2	0	granulated	A	8	M	[VIII: 3-4-3-4-3-4-2-3]	tuberculatus
<i>Isohypsibius monoicus</i> Bertolani 1982	P	3	0	rugose	A	0	—	—	?
<i>Isohypsibius monstrosus</i> Maucci 1991	A	2	0	granulated	A	10	E	[X: 2-2-6-4-6-4-6-4-2-2]	tuberculatus
<i>Isohypsibius montanus</i> Mihelčič 1938	P	2	0	smooth	P	?	M	?	tuberculatus
<i>Isohypsibius myrops</i> du Bois-Reymond Marcus 1944	A	3	0	smooth	A	0	—	—	prosostomus

Taxon	Eyespot Pigmentation	Macro-placoid Number	Micro-placoid Number	Cuticular Sculpturing	Tubercles	Number of Gibbosities Rows	Gibbosities Per Row	Gibbosities Formula	Species Group Assignment
<i>Isohypsibius neoundulatus</i> Durante Pasa & Maucci 1975	P	2	0	reticulated	A	6	E	[VI: 2-2-2-2-2-2]	undulatus
<i>Isohypsibius nipponicus</i> Sudzuki 1975	A	2	0	?	A	0	—	—	?
<i>Isohypsibius nodosus</i> Murray 1907b	P	2	0	granulated	A	7	E	[VII: 6-6-6-6-6-4]	tuberculatus
<i>Isohypsibius novaeguineae</i> Iharos 1967	P	2	0	granulated	A	9	O	[IX: 3-5-3-5-3-5-3-3-3]	tuberculatus
<i>Isohypsibius palmai</i> Pilato 1996	A	3	0	reticulated	A	0	—	—	?
<i>Isohypsibius panovi</i> Tumanov 2005	P	2	0	smooth	A	0	—	—	?
<i>Isohypsibius papillifer papillifer</i> Murray 1905	P	3	0	?	A	12	E	[XII: 2-2-2-4-6-6-6-6-6-2-4-2]	tuberculatus
<i>Isohypsibius papillifer bulbosus</i> Marcus 1928	P	3	0	?	A	11	E	[XI: 2-2-6-6-6-6-6-6-4-4-2]	tuberculatus
<i>Isohypsibius papillifer indicus</i> Iharos 1969 ^a	P	3	0	?	A	9	M	[IX: 4-6-4-6-4-6-4-4-3]	tuberculatus
<i>Isohypsibius pappi</i> Iharos 1966a	P	2	0	reticulated	A	10	E	[X: 2-4(2)6-4(2)6(2)4(2)6(4)4(2)4-2]	tuberculatus
<i>Isohypsibius pauper</i> Mihelčič 1971b	A	2 or 3	0	complex	A	0	—	—	?
<i>Isohypsibius pilatoi</i> Durante Pasa & Maucci 1979	A	2	0	reticulated	A	10	M	[X: 3-3-5-5-5-5-5-4-2-3]	tuberculatus
<i>Isohypsibius pratensis</i> Iharos 1964	P	2	0	reticulated	A	9	E	[IX: 2-4-6-4-4-2-6-4-4]	tuberculatus
<i>Isohypsibius prosostomus prosostomus</i> Thulin 1928	P	3	1	smooth	A	0	—	—	prosostomus
<i>Isohypsibius prosostomus cambrensis</i> Morgan 1976	P	3	1	granulated	A	0	—	—	prosostomus
<i>Isohypsibius pseudoundulatus</i> da Cunha & do Nascimento Ribeiro 1964	P	2	0	?	A	?	—	—	undulatus
<i>Isohypsibius pulcher</i> Mihelčič 1972	P	2	1	polygons	A	?	—	—	?
<i>Isohypsibius pushkini</i> Tumanov 2003a	P	3	0	granulated	A	0	—	—	tuberculatus
<i>Isohypsibius qinlingensis</i> Li, Wang & Yu 2005	A	2	0	?	A	8	M	[VIII: 3-2-3-2-3-2-2-2]	tuberculatus
<i>Isohypsibius rahmi</i> Li & Wang 2006	A	3	0		A?	10	M	[X: 4-4-4-4-4-2-4-4-2-3]	tuberculatus
<i>Isohypsibius reticulatus</i> Pilato 1973	P	2	0	reticulated	A	0	—	—	reticulatus
<i>Isohypsibius roberti</i> Biserov 1996	P	2	0	reticulated	A	0	—	—	elegans
<i>Isohypsibius ronsisvallei</i> Binda & Pilato 1969	P	2	0	reticulated	A	8	M	[VIII: 2-3-2-3-2-3-2-2]	tuberculatus
<i>Isohypsibius rudescui</i> Iharos 1966a	P	2	0	?	A	10	E	[X: 2-2-4-2-4-4-4-4-4-2]	tuberculatus
<i>Isohypsibius rugosus</i> Guidi & Grabowski 1996	A	3	0	irregular lines	A	0	—	—	granulifer
<i>Isohypsibius rusticus</i> Pilato, Sabella, & Lisi 2015	P	2	0	?	P	0	—	—	annulatus
<i>Isohypsibius sabellai</i> Pilato, Binda, Napolitano & Moncada 2004	P	2	0	reticulated	A	0	—	—	undulatus
<i>Isohypsibius sattleri</i> Richters 1902	P	3	0	reticulated	A	9	E	[XI: 2-2-2-4-6-6-6-2-4-4-4]	tuberculatus
<i>Isohypsibius schaudinni</i> Richters 1909	P	3	1	?	A	0	—	—	prosostomus
<i>Isohypsibius sculptus</i> Ramazzotti 1962	P	2	0	granulated	A	0	—	—	?
<i>Isohypsibius sellnicki</i> Mihelčič 1962	P	2	0	granulated	A	?	?	?	tuberculatus
<i>Isohypsibius septentrionalis</i> Thulin 1928	P	2	0	reticulated	A	6	O	[VI: 3-5-3-5-3-5]	tuberculatus

Taxon	Eyespot Pigmentation	Macro-placoid Number	Micro-placoid Number	Cuticular Sculpturing	Tubercles	Number of Gibbosities Rows	Gibbosities Per Row	Gibbosities Formula	Species Group Assignment
<i>Isohypsibius silvicola</i> Iharos 1966a§	P	2	0		A	10	M	[X: 2-2/4-4-4(1)-4(1)-4(1)-4(1)-4-4-3]	tuberculatus
<i>Isohypsibius sismicus</i> Maucci 1978	P	2	0	granulated	A	0	—	—	?
<i>Isohypsibius solidus</i> Mihelčič 1971b	A	2	0	smooth	A	0	—	—	prosostomus
<i>Isohypsibius taibaiensis</i> Li & Wang 2005	A	3	1	smooth	A	0	—	—	?
<i>Isohypsibius tetradactyloides</i> Richters 1907	P	3	0	smooth	A	0	—	—	?
<i>Isohypsibius theresiae</i> Iharos 1964	P	2	0	reticulated	A	8	E	[VIII: 4-4-4-4-4-4-2]	tuberculatus
<i>Isohypsibius torulosus</i> Mihelčič 1959	A	2	0	reticulated	A	10	M	[X: 3-2-6-4-(2)6-(6)6-4-(2)4-(4)6-3-(2)]	tuberculatus
<i>Isohypsibius truncorum</i> Iharos 1964	P	2	0	granulated	A	8	O	[VIII: 5-5-5-5-5-5-3-3]	tuberculatus
<i>Isohypsibius tuberculatus</i> Plate 1888	P	2	0	densely granulated, forming a reticulation	A	10	M	[X: 5-4-6-4-6-4-6-4-2-5]	tuberculatus
<i>Isohypsibius tuberculoides</i> Mihelčič 1951	P	2	0	?	A	9	M	[IX: 4-6-6-4-6-6-6-5-4]	tuberculatus
<i>Isohypsibius tubereticulatus</i> Pilato & Catanzaro 1989	A+P	3	0	reticulated	A	0	—	—	reticulatus
<i>Isohypsibius tucumanensis</i> Claps & Rossi 1984	P	2	0	scallops	A	10	M	[X: 4-2-5-5-5-5-5-4-4-2]	undulatus
<i>Isohypsibius undulatus</i> Thulin 1928	P	2	0	reticulated	A	0	—	—	undulatus
<i>Isohypsibius vej dovskyi</i> Bartoš 1939	?	2	0	granulated	P	9	E	[IX: 4-4-4-4-4-4-4-4-4]	tuberculatus
<i>Isohypsibius verae</i> Pilato & Catanzaro 1989	?	2	0	smooth	A	0	—	—	?
<i>Isohypsibius verrucosus</i> Della Valle 1915†	?	?	?	?	?	?	?	?	?
<i>Isohypsibius wilsoni</i> Horning, Schuster & Grigarick 1978	A+P	3	0	reticulated	A	0	—	—	?
<i>Isohypsibius woodsae</i> Kathman 1990	P	2	0	reticulated	A	10	E	[X: 4-4-4-4-4-4-4-4-4-2]	tuberculatus
<i>Isohypsibius yunnanensis</i> Yang 2002	P	3	0	“poriform”	A	0	—	—	?
<i>Isohypsibius zappalai</i> Pilato, Sabella, & Lisi 2015	P	2	0	?	P	0	—	—	annulatus
<i>Isohypsibius saulrogersi</i> sp. nov.	P	3	0	reticulated	A	10	E	[X: 4-6-4-6-6-6-2-4-4-4]	tuberculatus

* Difference between original description and the original figure with respect to gibbosities, row 9 also erroneously omitted.

§ Original description recorded either 2 or 4 gibbosities in row 2. Smaller, lateral ancillary swellings between rows indicate with numerals in parentheses.

† *sensu* Della Valle 1915, but needs redescription, nec *Calohypsibius verrucosus* (Richters, 1900)

Table 2.2. Summary of measurements for *Isohypsibius saulrodgersi* sp. nov.

Character	N	Holotype		Type Series Mean		Type Series Range	
		µm	pt	µm	pt	µm	pt
Body length	9	188.4	960.7	149.6	819.3	106.2–196.2	682–960.7
Buccal tube length	9	19.6		18.0		13.3–22.2	
Stylet support insertion point	7	12.5	63.8	12.0	62.9	9.2–14.5	58.8–65.6
Buccal tube external width	6	2.2	11.2	2.1	11.4	1.7–2.4	10.7–13.0
Buccal tube internal width	6	1.8	9.1	1.5	8.2	1.2–1.8	7.3–9.4
Placoid Lengths							
Macroplacoid 1	6	1.6	8.2	1.7	9.7	1.4–2.1	8.2–11.9
Macroplacoid 2	6	1.6	8.2	1.7	9.7	1.4–2.1	8.2–11.9
Macroplacoid 3	6	1.6	8.4	2.1	12.0	1.6–2.6	8.4–14.5
Macroplacoid row	5	6.9	35.0	6.9	39.8	6.4–8.3	35.0–42.9
Claw 1 lengths							
External base	5	1.3	6.5	0.9	5.2	0.5–1.3	3.9–6.5
External primary branch	6	7.9	40.2	7.6	42.3	5.8–8.6	39.0–48.4
External secondary branch	6	5.5	27.9	4.8	28.6	4.0–5.6	25.8–30.9
Internal base	6	0.9	4.8	0.8	4.1	0.6–1.0	3.6–4.8
Internal primary branch	5	6.1	31.2	6.6	33.6	5.9–7.6	31.2–36.7
Internal secondary branch	7	4.5	23.0	4.7	27.0	3.8–5.7	23.0–33.7
Claw 2 lengths							
External base	4	1.2	6.0	1.0	5.1	0.8–1.2	4.2–6.0
External primary branch	3	8.0	40.9	7.9	43.1	7.7–8.0	40.2–48.0
External secondary branch	3	4.9	25.1	4.9	25.3	4.6–5.2	24.0–26.7
Internal base	2			0.7	3.9	0.7–0.8	3.6–4.1
Internal primary branch	2	7.2	36.9	6.8	38.2	6.4–7.2	36.9–39.6
Internal secondary branch	3	5.4	27.4	4.7	24.8	4.0–5.4	21.9–27.4
Claw 3 lengths							
External base	5	0.9	4.6	1.1	5.5	0.9–1.2	4.4–7.4
External primary branch	3	7.6	39.0	7.7	38.2	7.1–8.5	36.1–39.5
External secondary branch	4	5.3	27.0	5.7	27.3	5.2–6.8	25.1–30.7
Internal base	3			0.8	4.3	0.7–0.9	3.5–4.8
Internal primary branch	4	6.7	34.1	7.1	34.1	6.7–8.1	31.3–36.6
Internal secondary branch	5	4.1	20.8	5.1	25.7	4.1–6.3	20.8–28.4
Claw 4 lengths							
External base	4			0.9	5.0	0.7–1.0	4.6–5.4
External primary branch	5	7.5	38.4	7.9	37.2	7.1–8.8	34.7–38.7
External secondary branch	5	5.5	28.0	5.7	29.9	4.4–7.0	27.2–32.9
Internal base	3			0.7	2.6	0.5–1.0	2.5–2.6
Internal primary branch	5	6.7	34.0	7.0	33.3	6.6–7.6	29.8–36.6
Internal secondary branch	6	5.1	25.9	5.0	26.6	3.9–5.9	24.7–29.3

— unsuitable for measurement

Figure 2.2. Holotype, *Isohypsibius saulrodgersi* sp. nov., (drawn from phase contrast microscope: PhC).

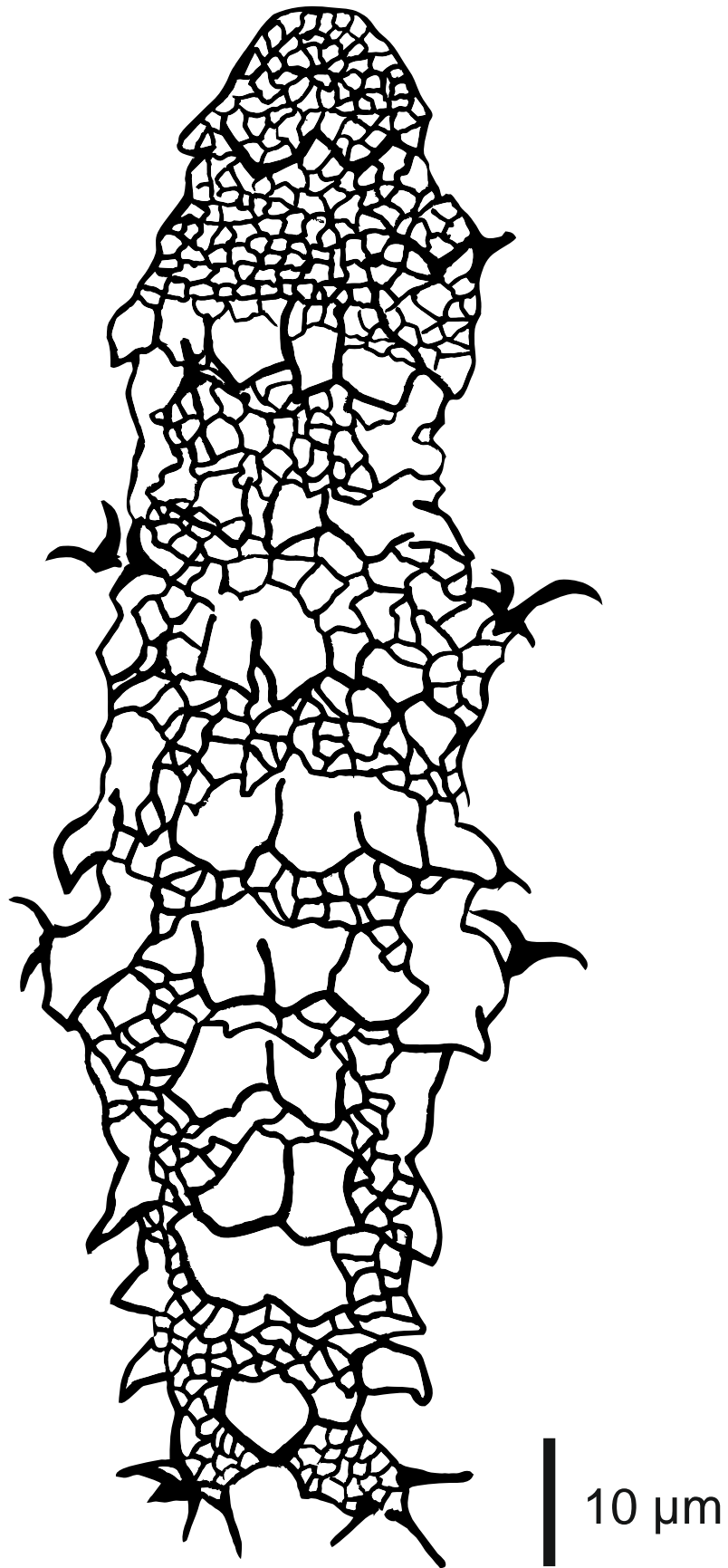


Figure 2.3. Large-scale schematic plan of *Isohypsibius saulrodgersi* showing the arrangement of gibbosities.

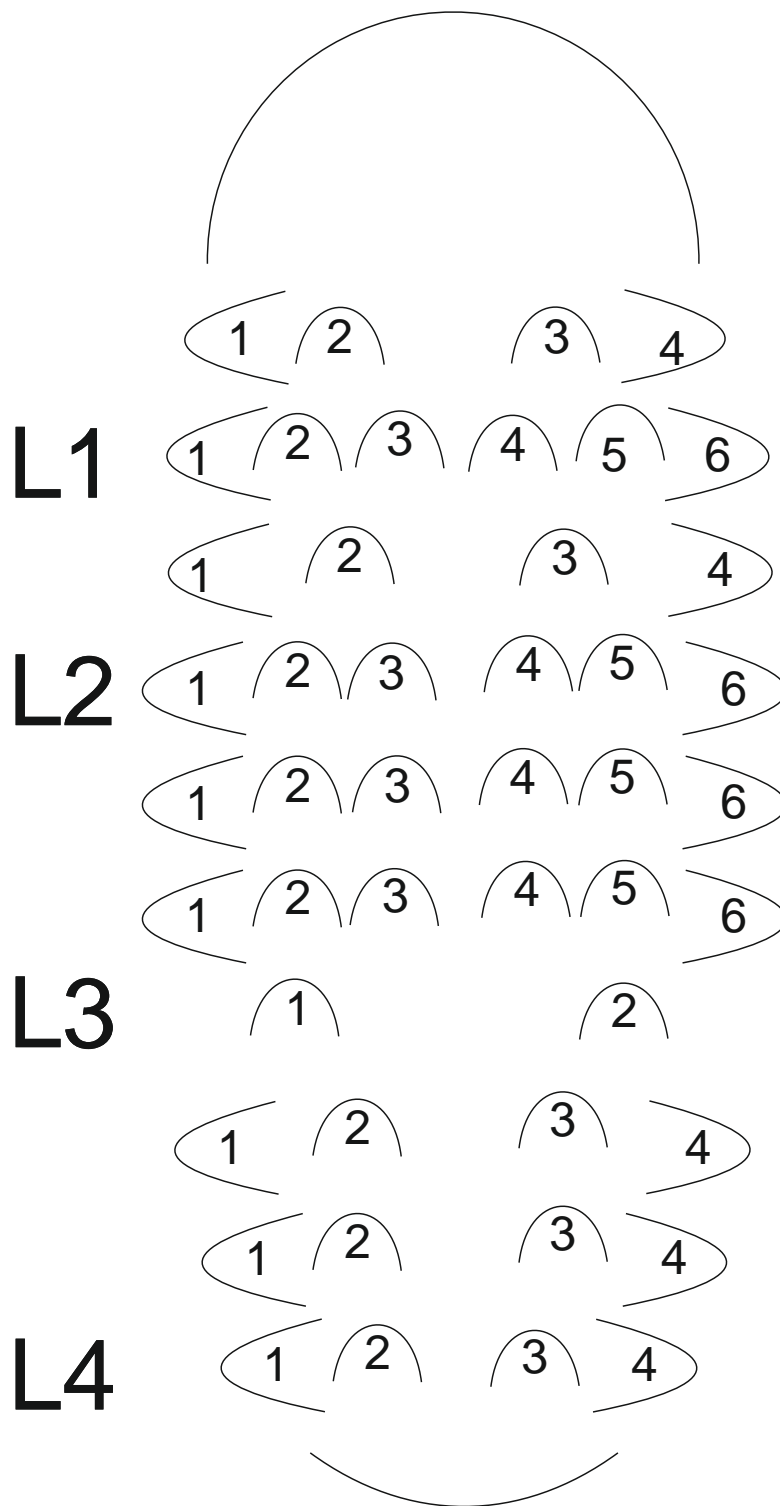


Figure 2.4. Holotype, *Isohypsibius saulrodgersi* sp. nov., A) dorsal gibbosities, B) buccopharyngeal apparatus, C) claws of leg pairs I–VI. DIC.

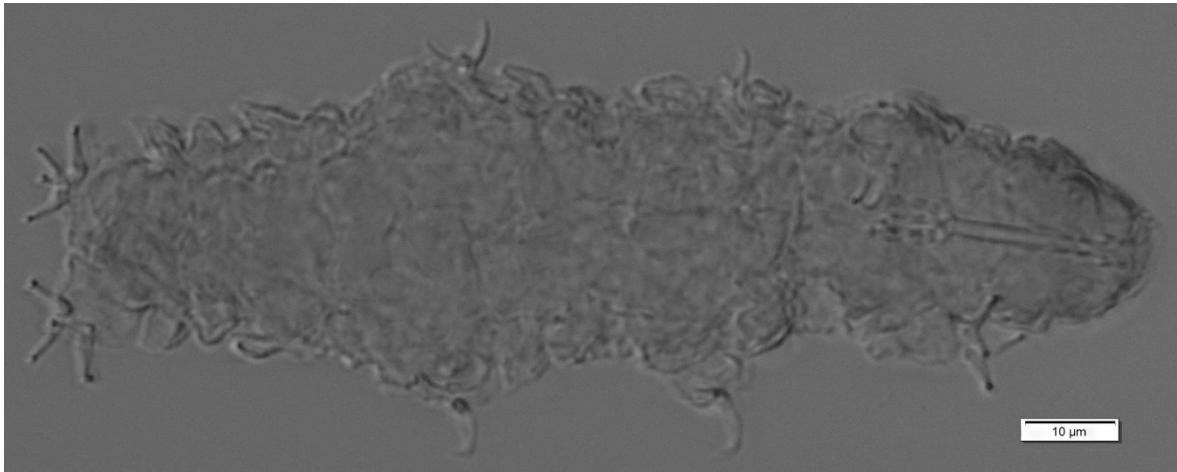
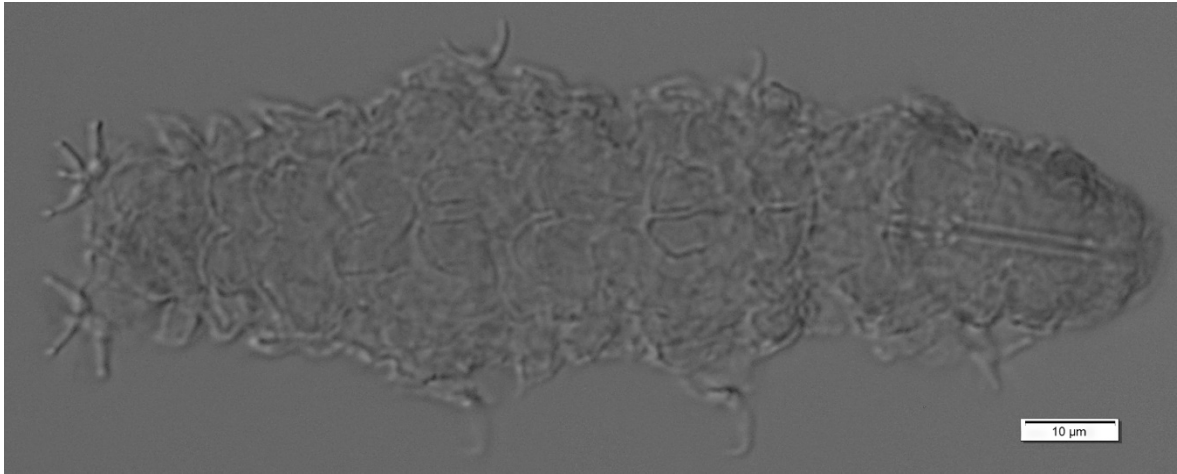


Figure 2.5. Paratype 2, *Isohypsibius saulrodgersi* sp. nov., A) claws leg I, B) claws leg II, C) Claws leg III, D) Claws leg pairs IV and dorsal gibbosities, E) buccopharyngeal apparatus. Photos taken under DIC.

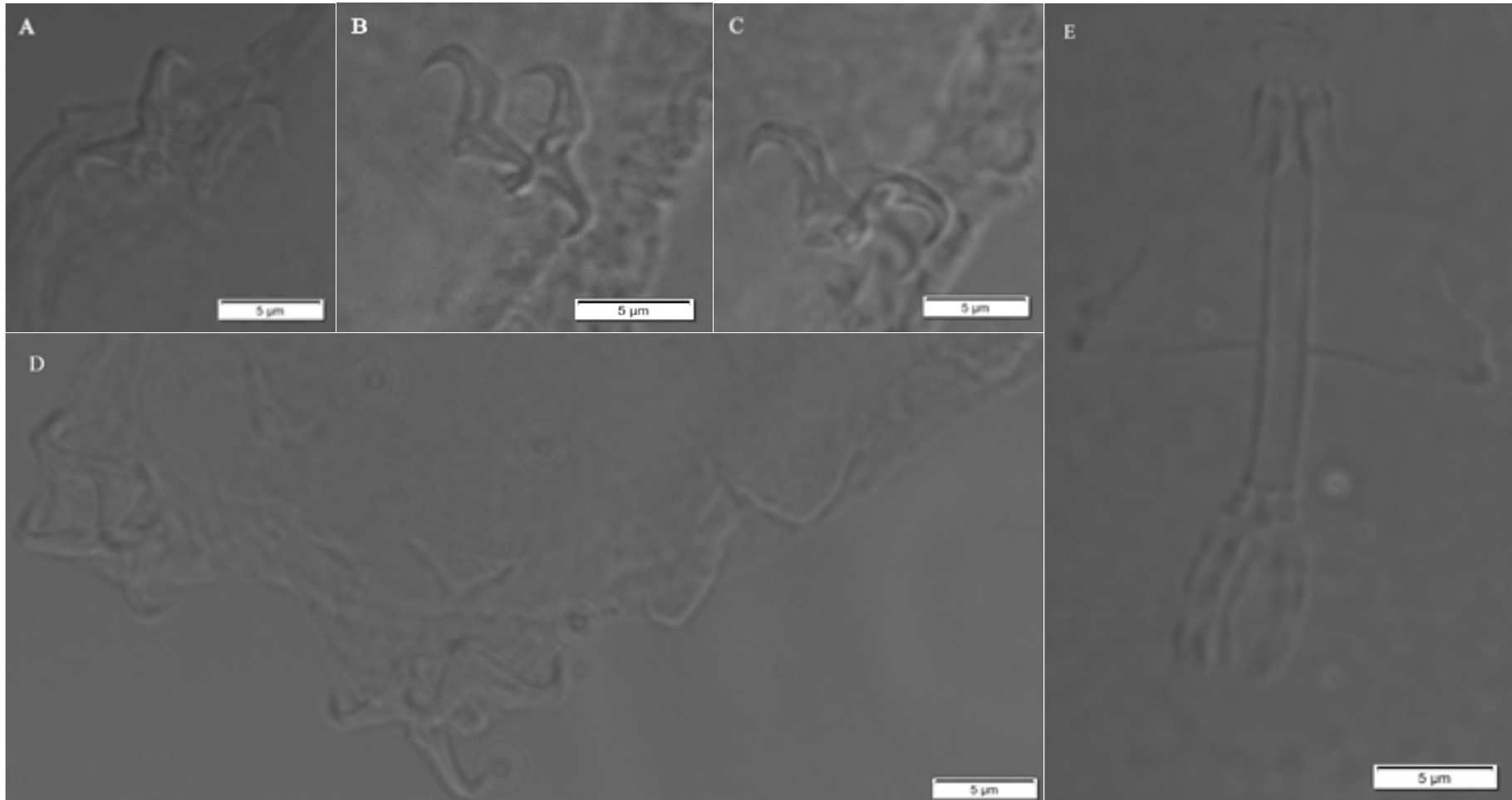
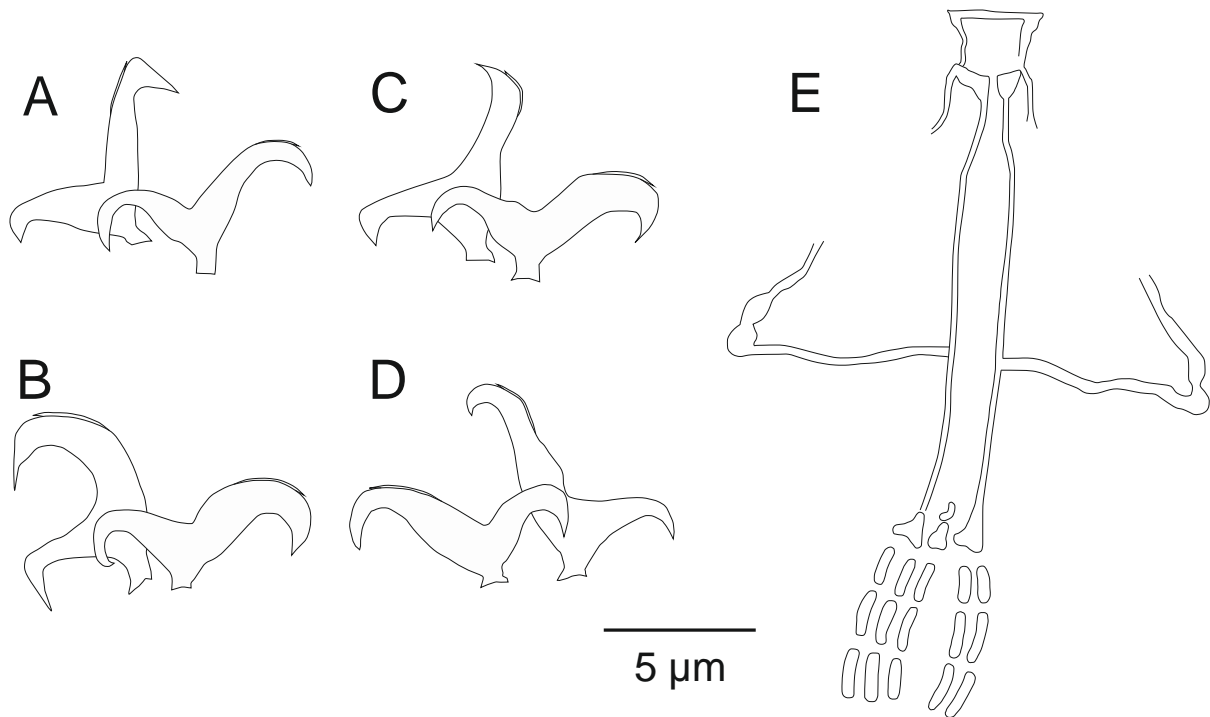


Figure 2.6. Paratype, *Isohypsibius saulrodgersi* sp. nov., A) claws leg I, B) claws leg II, C) claws leg III, D) claws leg IV, and E) buco-pharyngeal apparatus. Photos taken under DIC.



Remarks

In the same samples, 31 other tardigrade taxa were found, mostly belonging to genera *Adropion*, *Diphascon*, *Echiniscus*, *Hypsibius*, *Isohypsibius*, *Macrobiotus*, *Milnesium*, *Minibiotus*, *Paramacrobiotus*, *Platicrista* and *Ramazzottius*. Several nematodes and rotifers were also found.

Photographs and drawings from a paratype specimen are presented in Figs 2.5 and 2.6.

Type material

The holotype is deposited in the QCAZ Museum of the Pontificia Universidad Católica del Ecuador, Quito (accession number QCAZI 3443). There are nine paratypes and these will be deposited into the following collections: seven specimens will remain in the first two authors' collection (Nigel Marley and Balbina Ramsay) at Plymouth University, UK, one specimen will be sent to Dr R. Guidetti, Università degli Studi di Modena e Reggio Emilia, Modena, Italy, one specimen will go to Dr Ł. Kaczmarek, at Adam Mickiewicz University, Poznań, Poland.

Type locality

The specimens were collected from the ground in a fragmented woodland at 3,575 masl, dominated by trees of *Polylepis sericea* Wedd., just outside El Angel Ecological Reserve,

Carchi Province, northern Ecuador (UTM coordinates 18 N 168316 78347; latitude and longitude N 0° 42'28.3" W 77° 58'46.7").

Etymology

The species is named after Mr Saul Rodgers, a friend of one of the first author (Balbina Ramsay).

Differential diagnosis

Isohypsibius is a species rich genus consisting of 133 taxa (Degma *et al.* 2017). Seven other *Isohypsibius* species shared several characteristics with the new species, while another seven species shared other characteristics:

1. Cuticle not smooth (*i.e.*, sculptured or unknown), without tubercles, gibbosities present in ten rows, even number of gibbosities in all rows. The species in this group are: *Isohypsibius bartosi* (Iharos, 1966); *Isohypsibius brevispinosus* (Iharos, 1966a); *Isohypsibius josephi* Iharos, 1964; *Isohypsibius monstruosus* (Maucci, 1991); *Isohypsibius pappi* (Iharos, 1966a); *Isohypsibius rudescui* (Iharos, 1966a); and *Isohypsibius woodsae* (Kathman, 1990).
2. Macroplacoid numbers either three or two/three in the original description, no microplacoid, no septulum, tubercles absent, between nine and eleven rows of gibbosities, with an even number of gibbosities per row. The species in this group are: *Isohypsibius brevispinosus* (Iharos, 1966a); *Isohypsibius costatus* (Mihelčič, 1971); *Isohypsibius franzi* Mihelčič, 1951; *Isohypsibius josephi* Iharos, 1964; *Isohypsibius mammosus* Iharos, 1964; *Isohypsibius papillifer bulbosus* Marcus, 1928; and *Isohypsibius sattleri* Richters, 1902.

I carried out differential diagnoses on these two sets of species, based on the character matrix:

Set 1

I. bartosi has a different type of cuticular sculpturing (granulated with gibbosities). It has just two macroplacoids. The arrangement and number of gibbosities differs: [X: 4-4-4-4-4-4-4-4-2].

I. brevispinosus differs in its type of cuticular sculpturing (granulated). It has the same number of macroplacoids (three). The arrangement and number of gibbosities differs: [X: 6-4-4-6-4-4-6-4-4].

I. josephi has been described with two different arrangements of gibbosities (one falls into this set of similar species, while the other arrangement falls into the second set, described later). It has just two macroplacoids. The original description is not clear about the detail of cuticular sculpturing on the dorsum. The arrangement and number of gibbosities differs: [X: 2-2-4-4-4-4-4-4-4-4].

I. monstruosus has a different type of cuticular sculpturing (granulated with gibbosities), and has just two macroplacoids. The arrangement and number of gibbosities differs: [X: 2-2-6-4-6-4-6-4-2-2].

I. pappi has the same type of cuticular sculpturing (reticulated), but has just two macroplacoids. The arrangement and number of gibbosities differs: [X: 2-4-6-4-6-4-6-4-4-2], with ancillary lateral gibbosities between rows 2 and 3, 4 and 5, 5 and 6, 6 and 7, 7 and 8, and 8 to 9.

I. rudescui has a different type of cuticular sculpturing (granulated with gibbosities), and has just two macroplacoids. The arrangement and number of gibbosities differs: [X: 2-2-4-2-4-4-4-4-4-2].

I. woodsae has the same type of cuticular sculpturing (reticulated), but has just two macroplacoids. The arrangement and number of gibbosities differs: [X: 4-4-4-4-4-4-4-4-4-2].

Set 2

I. costatus has a different type of cuticular sculpturing (granulated with gibbosities). The number of macroplacoids varies between two and three. The arrangement and number of gibbosities differs: [XII: 2-2-2-4-3-4-4-2-4-4-5-2]

I. franzi has no cuticular sculpturing (smooth). The number of macroplacoids varies between two and three. The arrangement and number of gibbosities differs: [IX: 6-6-6-6-6-6-4-4-4].

I. josephi, according to the alternative description, has an unknown type of cuticular sculpturing. It has just two macroplacoids. The arrangement and number of gibbosities differs: [IX: 2-4-4-4-4-4-4-4-2].

I. mammillosus has a different type of cuticular sculpturing on the dorsum (granulated). It has the same number of macroplacoids (three). The arrangement and number of gibbosities differs: [XI: 4-4-4-4-4-4-4-4-4-2].

I. papillifer bulbosus has an unknown type of cuticular sculpturing. It has the same number of macroplacoids (three). The arrangement and number of gibbosities differs: [XI: 2-2-6-6-6-6-6-4-4-4-2].

I. sattleri has a different type of cuticular sculpturing (reticulated). It has the same number of macroplacoids (three). The arrangement and number of gibbosities differs: [XI: 2-2-2-4-6-6-6-2-4-4-4].

It is clear from these comparisons that the most similar taxa to the new species are nevertheless different in several important respects. Thus, I am confident in describing our specimens as a new species to science within this species-rich genus.

Discussion

Isohypsibius saulrogersi sp. nov. differs from all other described species within the genus in several ways. The arrangement and number of gibbosities is unique (in comparison to with current list of species), and other characters, in combination, also differentiate it from those other species. These characters include cuticular sculpturing, the number of macroplacoids, the absence of microplacoids, and the presence of tubercles. The species is added to the relatively small number of tardigrade species so far identified from the high-altitude *Polylepis* forests of the Andes, though many more remain to be determined. It is possible that this new species is a specialist in tropical mountain forests, but it could be present in a wider range of habitats in the Andes. More sampling would be needed to determine this.

Traditionally, dichotomous keys have been used by experienced researchers to identify both described taxa and to identify potentially new taxa. However, if one is not an expert with the specific taxonomic group, using these dichotomous keys can be problematic, requiring educated guesses if a character is not visible on a specimen. I wanted to develop a character matrix to provide a potential solution to this problem.

Isohypsibius provided an ideal case study, because it contains a large number of taxa but a limited number of taxonomic characters, and restricted type material with relatively old taxonomic descriptions with incomplete details. In developing and using this

approach I encountered several important issues, with probable relevance to the application of this approach to other taxonomic groups.

Type material no longer exists for some taxa. For example, the collection of Dr Mihelčič, including type specimens, was lost when the Tiroler Landesmuseum Ferdinandeum was flooded in 1985 (Dastyč 1993). In addition, some material which does exist is not easily available for loan, such as material from Chinese collections. In these cases, I was forced to rely completely on the published descriptions and illustrations.

A common problem with older publications was that the original description was limited by modern standards. For example, Ramazzotti (1945a) described *I. baldii* and emphasised cuticular characteristics but gave few details about the claws or buccal apparatus. As more new species were described over time, the formal descriptions tended to include more details about a wider range of characteristics since they were needed to differentiate the new species from ones already described. However, even some modern descriptions sometimes lack sufficient detail. For example, Yang's (2002) description and differential diagnosis for *I. yuannensis* provides little information about essential characteristics such as the buccal apparatus or claws. His translation in the differential diagnosis within the paper merely states, "Lijiang specimens differ from all of the known species in major aspects. So, considered as new to science."

Likewise, original illustrations are rather variable in quality and content. They do not always show the required details, and sometimes they do not even match the text description given in the same paper. In Mihelčič (1951), the arrangements of gibbosities for both *I. franzi* and *I. dudichi* were described differently in the text from the way they were illustrated (I followed the illustration). Iharos (1964) did not describe the gibbosities for *I. josephi* with sufficient clarity, and I was forced to consider this species independently in two differential diagnoses to account for the potential alternative characteristics.

I also needed to consider carefully the terminology used in the publications, especially in translation, because sometimes definitions vary from description to description. Occasionally, I needed to use my judgement to interpret the original wording in descriptions and match them with the categories in our character matrix. For example, the cuticle of *I. monoicus* (Bertolani 1982) was originally described as "Persian lamb-like", which I interpreted to mean "rugose".

Constructing the character matrix was, therefore, a complex and long task, involving literature in many languages and from a wide range of sources. However, once it was completed, it provided a very rapid and reliable reference for confirming character relationships between specimens and described species. I could quickly eliminate large numbers of potential taxa as a match for our new species because they failed to match particular character states. In all cases, I could eliminate species using several different characters independently, increasing confidence. These tasks were made simpler by using filters with a spreadsheet version of Table 1 (available as Supplementary Material 1). At the same time, this process provided a clear basis for differential diagnoses of similar species.

Conclusions

The use of a character matrix simplified the process of determining our specimens as belonging to a new species, *Isohypsibius saulrogersi* sp. nov. Since Ecuadorian Tardigrada remain relatively unknown, especially in high-altitude habitats such as *Polylepis* forest, I would recommend this approach for other tardigrade taxa in order to accelerate the description of new species, and to incorporate these new species into a user-friendly descriptive framework.

One advantage of developing a character matrix for a particular taxonomic group is that it provides a clear statement of the range of characters that have been used to describe the species within a taxon. It should, therefore, help to avoid descriptions which miss out crucial characters. It also helps to standardise the way descriptions of particular characters are worded, so that direct comparisons can be made more easily.

Another clear advantage lies in the ability to narrow down quickly the field of potential matches between specimens and described taxa, using characters in any order. Even if certain characters are not visible in a particular specimen, the combination of other characters might be enough to provide a confident match. Furthermore, the inherent redundancy within a character matrix provides potential tolerance of errors from the user: an error associated with one character will often be compensated by the remaining combination of characters, making misdiagnosis less likely.

On the other hand, a character matrix should have the potential to evolve through time (as written descriptions and illustrations have) to include character sets and character states which have yet to emerge, but which prove important in the future. An existing

matrix should not pressure taxonomists into describing new species in a constrained manner just to fit better with the matrix characters. Rather, the character matrix should outline a contemporary minimum standard with room for growth.

3 The effect of microhabitat on the distribution of tardigrades at high-altitude forest in the Peruvian Andes

Introduction

In most habitats, micrometazoans, organisms less than 2 mm in size (Guil, 2002), represent a poorly understood, but apparently significant component of overall biodiversity (Hunter-Cevera, 1998, Øvreås, 2000, Fontaneto *et al.*, 2006). However, despite their likely high species richness and abundance, surveys of these smaller invertebrates are scarce, probably due to a lack of basic taxonomic expertise for many groups (McInnes, 1994, Rundle *et al.*, 2000)

Tardigrades are an important group of micrometazoans, many of which have extraordinary resistance to physical and chemical extremes (Wright, 2001). They are abundant and speciose in a wide range of situations from the Equator to the Poles, and in both aquatic and terrestrial environments (Kathman and Cross, 1991, Sanchez-Moreno *et al.*, 2008, Guil and Sanchez-Moreno, 2013). With a body size range between 50–1200 µm (Dewel *et al.*, 1993), these animals fall in a size category at which, it has been argued, organisms change from being cosmopolitan to having potentially restricted distributions (Finlay and Fenchel, 2004). For a micrometazoan group they are also relatively convenient to work with because they are easy to collect and store, partly due to their ability to survive desiccation and extreme or significant temperature variations (Wright, 2001). Nevertheless, there is a lack of distributional data for tardigrades due to under sampling in most parts of the world (McInnes, 1994, Fontaneto *et al.*, 2006), including South America, something which limits understanding of the factors driving assemblage composition and global distributions (Fontaneto *et al.*, 2005, Fontaneto *et al.*, 2006).

The Andes of South America provide a useful natural laboratory for studying the biogeography of tardigrades. In addition to altitudinal variation, the north-south orientation of the mountain chain allows the study of distributions across a wide range of latitudes. Forests dominated by trees belonging to the genus *Polylepis* are found throughout the Andes from Venezuela and Colombia in the north to Chile and Argentina in the south (Fjeldså and Kessler, 1996). *Polylepis* forests provide a variety of microenvironments which differ from those in surrounding páramo grasslands:

conditions inside are more humid and temperatures are lower and fluctuate less than outside forests (Ramos *et al.*, 2013). However, conditions also vary within the forest structure itself, since canopies receive more light, are drier, and are subjected to wider ranges of temperature than the forest floor (Lowman and Rinker, 2013). The trunks of trees can modify water chemistry (such as pH) through the close contact of water with the bark (Bates, 1992). Thus, micrometazoans within the forest can experience different environments depending on their position within the forest structure, and so their distribution patterns are likely to vary according to forest structural location.

In the same way that tree bark can modify water chemistry, different substrates can also affect conditions at the fine scale. For example, rocks and soil create different local conditions, especially regarding water availability and chemistry (Bates, 2008), which might favour particular tardigrade taxa.

Tardigrades can be found in a variety of hosts, such as bryophytes (mosses, liverworts and hornworts), lichens, algae, and vascular plants (Horning *et al.*, 1978, Bertolani and Rebecchi, 1996, Ito, 1999, Hinton and Meyer, 2007, McFatter *et al.*, 2007, GuilSanchez-Moreno *et al.*, 2009). The structural complexity of these hosts has been linked to micrometazoan diversity (Gradstein *et al.*, 2001), but also host chemistry might be important (Glime, 2006). Furthermore, the hosts themselves also respond to microclimatic conditions within the forest structure and on different substrates (Bates, 2008).

The aims of this study were to examine patterns of tardigrade distribution and abundance driving small-scale differences in assemblage composition and microhabitat use, by sampling tardigrades from different positions within a *Polylepis* forest and the surrounding grassland, as well as on different hosts and substrates.

Materials and methods

The study area was located in the valley of Mantamay, situated in the Cordillera de Vilcanota, approximately 40 km NNW of Cusco, Peru (Fig. 3.1). It is located relatively centrally in the latitudinal range of the Andes, and therefore provides a useful starting point for exploring biogeographical patterns in *Polylepis* woodlands. The valley contains several large patches of *Polylepis* forest as well as small fragments, across a range of altitudes from 3,800 to 4,800 m above sea level. This study focused on one large patch of forest and puna grassland at 4150 m (coordinates 13° 12' 35" S 72° 9' 50" W).

Knowledge of tardigrades from this region is very limited and only 28 taxa have been reported for Peru (Murray and Wailes, 1913, Marcus, 1939, Binda and Pilato, 1995, Pilato, 2000, Nickel *et al.*, 2001, Pilato *et al.*, 2001, Michalczyk and Kaczmarek, 2003, Michalczyk and Kaczmarek, 2004, Pilato *et al.*, 2004, Michalczyk and Kaczmarek, 2006, Kaczmarek *et al.*, 2014). With the exception of Marley (2006), who described a tardigrade species from the Ecuadorian Andes, no studies exist from similar ecosystems in the Andes.

Figure 3.1. Location of the study site in the Valley of Mantamay, Cordillera de Vilcanota, Peru.



Tardigrades were sampled along a 135 m transect running from puna grassland, across the forest edge, and into the forest interior of a representative large patch of *Polylepis* forest and puna grassland at an altitude of 4,150 m. In total, 77 samples were collected. Each of these samples was categorised according to its “position” within the forest structure and neighbouring puna grassland (tree trunks, canopy branches, forest floor, grassland), the type of “host” (bryophytes, lichens, bark) and the “substrate” (soil, rock, tree).

The collection was made in August 2005, during the dry season when most lichens and bryophytes were dry. Samples were placed into paper envelopes, air dried and then stored at room temperature until they were processed. In the laboratory, the dry samples were weighed, and then rehydrated in water for at least 16 h. Hydrated samples were shaken and water, plus sediment, transferred into a 38 µm mesh sieve. The sieved content was transferred to a Petri dish, and examined at 30–40x under a Kyowa SDZ-PL stereoscopic microscope (Kyowa, Japan). Individual tardigrades were mounted on microscope slides with Hoyer's mounting medium. Tardigrades were identified to the level of OTUs (Operational Taxonomic Units) with a standard Leica DMLB microscope, using the current classifications of Guidetti and Bertolani (2005) and Marley *et al.* (2011). Some tardigrades found in this study were new taxa to science.

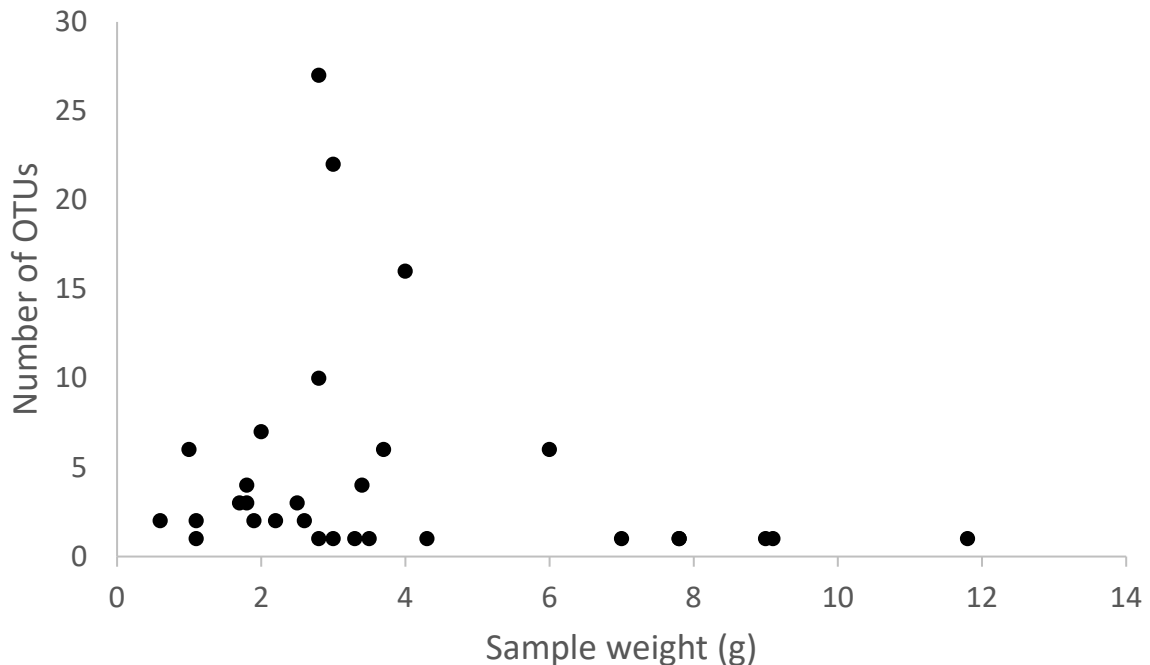
Differences between the numbers of tardigrades and tardigrade species found in samples from different positions, substrates and hosts were analysed using one-way General Linear Model (GLM ANOVA) with STATISTICA 10 (Stat Soft Inc., Tulsa, USA). Since the samples available did not provide a balanced design, it was not possible to consider interactions between position, substrate and host.

Differences in taxonomic composition of samples were assessed using non-metric Multidimensional Scaling (MDS) with Primer 6 (Primer-e, Plymouth, UK). The graphical output of this approach positions samples with similar composition close together and samples with very different composition far apart. Statistical differences in composition between sample categories (according to position, substrate and host) were determined with permutational ANOVA (PERMANOVA) using the PERMANOVA+ add-on to Primer 6. PERMANOVA does not assume the data are normally distributed (which they were not for our tardigrade samples) because it uses a permutational approach, making the analysis distribution-free. However, PERMANOVA is sensitive to differences in the dispersion of data (Anderson *et al.*, 2008) and so an additional test, when significant differences were identified by PERMANOVA, was carried out to identify any significant differences in dispersion between groups, using PRIMER's PERMDISP.

Results

A total of 77 samples were examined, 41% of which contained tardigrades. There was no relationship between sample weight and the number of individual tardigrades found within (Fig. 3.2).

Figure 3.2. There was no relationship between sample weight and the number of individual tardigrades within.



Samples contained 0 to 27 individuals, from up to 10 taxa. From a total of 139 specimens, 27 OTUs were identified, with higher taxon richness of eutardigrades compared with heterotardigrades (22 vs 5; Table 3.1). Eutardigrades were also more abundant than heterotardigrades (82 individuals vs 57). The eutardigrades in the forest were represented by the genera *Macrobiotus* (5 taxa), *Minibiotus* (5), *Hypsibius* (3), *Diphascon* (3), *Milnesium* (2), *Platicrista* (1), *Isohypsibius* (1), *Murrayon* (1). The forest heterotardigrades were represented by only two genera: *Echiniscus* (2 taxa) and *Pseudechiniscus* (3). Only one grassland sample contained tardigrades, all of which belonged to an apparently new species of *Calcarobiotus* (Eutardigrada; Table 3.1).

An initial multivariate analysis of tardigrade composition grouped most samples close together, with four outlying samples (Fig. 3.3). Each of these outlying samples contained a single, unique, taxon, none of which were found in any of the other samples. These outliers were therefore excluded from further compositional analyses described below.

In Fig 3.4, tardigrade taxa occupying the same samples occur close together, whereas taxa found in mutually exclusive samples are located further apart. Taxa around the periphery of the figure, therefore, tend to occur in samples with few other taxa (*e.g.*, *Minibiotus* sp.). By contrast, taxa in the centre of the figure tend to co-occur with several other taxa (*e.g.*, *Murrayon* sp.). There are no clusters of species, suggesting that taxa do not co-occur as groups of species specific to certain samples.

Inside the forest, tardigrades were present on branches, tree trunks and the forest floor (Table 3.1). There were no significant differences in tardigrade abundance or taxon richness between these positions within the forest (GLM ANOVA $F_{3, 73}=1.504$, $p=0.221$; $F_{3, 73}=2.086$, $p=0.109$). The only grassland sample with tardigrades (not included in the statistical tests) had just one individual. Samples from the forest floor differed in tardigrade composition from *Polylepis* branch samples (Fig. 3.5; permutational ANOVA $df=2$, Pseudo- $F=1.8009$, $p=0.022$; pairwise test $p=0.024$). The forest floor samples showed significantly greater dispersion than the branch samples (PERMDISP, $df=2$, $p=0.001$; pairwise test $p=0.002$). Trunk samples did not differ significantly in composition from forest floor samples (pairwise test $p=0.152$) or branch samples (pairwise test $p=0.101$).

Only two taxa were found exclusively in lichen samples, while 14 taxa were found exclusively in bryophytes samples (Table 3.2). However, there were no significant differences in tardigrade abundance or taxon richness between these two host types ($F_{2, 74}=1.130$, $p=0.328$; $F_{2, 74}=1.602$, $p=0.208$). One of four samples of papery *Polylepis* tree bark (not included in the statistical tests) contained a single individual (*Macrobiotus* sp. nov.). There were no differences in tardigrade composition between lichens and bryophytes (Fig. 3.6; PERMANOVA analysis $df=2$, Pseudo- $F=1.1058$, $p=0.312$).

Tardigrades were found on samples of soil, rock and tree (Table 3.3). Two taxa were only found on rock substrate, while six were found only on trees. All taxa found in soil samples (all from a single sample) were also found on other substrates. There were no significant differences in tardigrade abundance or taxon richness between rock and tree substrates ($F_{2, 74}=1.410$, $p=0.251$; $F_{2, 74}=1.677$, $p=0.194$). There were also no differences in tardigrade composition between samples from soil, rock and tree (Fig. 3.7; PERMANOVA analysis $df=2$, Pseudo- $F=1.1336$, $p=0.301$).

Table 3.1. The mean number of tardigrades in each species of the three different positions in the forest structure and grassland. “cf.” denotes OTUs which are similar to the species named, but have not been formally confirmed as that species. Numbers following a species or genus name indicate clearly recognisable, different, morphospecies. Some species belong to complex groups that have yet to be resolved taxonomically, and this is also indicated. “Simplex” refers to a taxon that could not be identified beyond genus level because it lacked visible features needed for identification. Authorities for species are given in Table 3.2.

Forest Position and Grassland						
Taxa	Trunk	Branch	Forest floor	Grassland	Overall	
	n=	40	6	28	3	77
Heterotardigrada						
<i>Echiniscus bigranulatus</i>			0.3	1.2		1.5
<i>Echiniscus cf. ollantaytamboensis</i>				<0.1		<0.1
<i>Pseudechiniscus cf. novaezeelandiae</i>			0.2			0.2
<i>Pseudechiniscus spinerectus</i>	<0.1	2.3	0.1			2.5
<i>Pseudechiniscus suillus</i>			0.1			0.1
Eutardigrada						
<i>Calcarobiotus</i> sp. nov					0.3	0.3
<i>Diphascon adropion</i> 311	<0.1	0.3				0.4
<i>Diphascon dastychy</i>	<0.1	0.3	0.1			0.4
<i>Diphascon victoriae</i>	<0.1					<0.1
<i>Hypsibius</i> sp.			0.1			0.1
<i>Hypsibius</i> sp. 200		0.8				0.8
<i>Hypsibius cf. valentinae</i>	<0.1	0.3	0.1			0.4
<i>Isohypsibius sattleri-bakonyiensis</i>		0.3	<0.1			0.3
<i>Macrobiotus cf. pseudohufelandi</i>			0.1			0.1
<i>Macrobiotus areolatus</i> group			0.2			0.2
<i>Macrobiotus hufelandi</i> group			0.4			0.4
<i>Macrobiotus simplex</i>			<0.1			<0.1
<i>Macrobiotus</i> sp. nov.	<0.1		<0.1			<0.1
<i>Milnesium brachyungue</i>	<0.1	0.5				0.5
<i>Milnesium</i> sp. nov.		0.2	<0.1			0.2
<i>Minibiotus constellatus</i>			0.1			0.1
<i>Minibiotus eichorni</i>		0.2				0.2
<i>Minibiotus</i> sp.	<0.1	1	0.2			1.2
<i>Minibiotus sidereus</i>		0.2				0.2
<i>Minibiotus simplex</i>			<0.1			<0.1
<i>Murrayon</i> sp.	<0.1		0.3			0.3
<i>Platicrista ramsayi</i>		0.2	<0.1			0.2
Mean number of individuals per sample	<0.1	0.5	0.2	0.3	0.3	

Table 3.2. The mean number of tardigrades of each taxon per sample of three different types of host (bryophyte, lichen, *Polylepis* tree bark).

Taxa	Host				Overall
	n=	Bryophyte	Lichen	Bark	
		54	19	4	77
Heterotardigrada					
<i>Echiniscus bigranulatus</i> (Richters 1907)		0.3	1.1		1.4
<i>Echiniscus cf. ollantaytamboensis</i> (Nickel Miller & Marley 2001)		<0.1			<0.1
<i>Pseudechiniscus cf. novaezeelandiae</i> group (Richters 1908)		<0.1			<0.1
<i>Pseudechiniscus spinerectus</i> (Pilato, Binda, Napolitano & Moncada 2001)		0.3	0.1		0.4
<i>Pseudechiniscus suillus</i> (Ehrenberg 1853)		<0.1	0.5		0
Eutardigrada					
<i>Calcaribiotus</i> sp. nov. (Dastych 1993)		<0.1			<0.1
<i>Diphascon cf. adropion</i> 311		<0.1			<0.1
<i>Diphascon dastychi</i> (Pilato & Binda 1999)		<0.1			<0.1
<i>Diphascon victoriae</i> (Pilato & Binda 1999)		<0.1			<0.1
<i>Hypsibius</i> sp. (Ehrenberg 1848)		<0.1	<0.1		<0.1
<i>Hypsibius</i> sp. 200 (Ehrenberg 1848)		<0.1	0.1		0.1
<i>Hypsibius cf. valentinae</i> (Pilato, Kiosya, Lisi & Sabella 2012)		<0.1			<0.1
<i>Isohypsibius sattleri-bakonyiensis</i> (Iharos 1964)		<0.1	<0.1		<0.1
<i>Macrobiotus cf. pseudohufelandi</i>		<0.1			<0.1
<i>Macrobiotus areolatus</i> group (Murray 1907)		0.1			<0.1
<i>Macrobiotus hufelandi</i> group (C.A.S. Schultze 1833)		0.2			0.2
<i>Macrobiotus simplex</i>		<0.1			<0.1
<i>Macrobiotus</i> sp. nov.		<0.1		0.3	0.3
<i>Milnesium brachyungue</i> (Binda & Pilato 1990)		<0.1	0.1		0.1
<i>Milnesium</i> sp. nov. (Doyère, 1840)		<0.1	<0.1		<0.1
<i>Minibiotus constellatus</i> (Michalczyk & Kaczmarek 2003)			0.2		0.2
<i>Minibiotus eichhorni</i> (Michalczyk & Kaczmarek, 2004)		<0.1			<0.1
<i>Minibiotus</i> sp. (R.O. Schuster 1980)		0.1	0.3		0.4
<i>Minibiotus sidereus</i> (Pilato, Binda & Lisi 2003)		<0.1			<0.1
<i>Minibiotus simplex</i>			<0.1		<0.1
<i>Murrayon</i> sp. (Bertolani & Pilato 1988)		0.2	<0.1		0.2
<i>Platicrista ramsayi</i> (Marley 2006)		<0.1	<0.1		<0.1
Mean number of individuals per sample		<0.1	0.2	0.3	0.1

Table 3.3. The mean number of tardigrades of each taxon per sample, on each of three different substrates.

Taxa	Substrate				Overall
	Soil	Rock	Tree		
	<i>n</i> =	3	28	46	
Heterotardigrada					
<i>Echiniscus bigranulatus</i>			1.2	<0.1	1.2
<i>Echiniscus cf. ollantaytamboensis</i>			<0.1		<0.1
<i>Pseudechiniscus cf. novaezeelandiae</i>			<0.1	<0.1	
<i>Pseudechiniscus spinerectus</i>			0.1	0.3	0.4
<i>Pseudechiniscus suillus</i>			<0.1		
Eutardigrada					
<i>Calcarobiotus</i> sp. nov	0.3				0.3
<i>Diphascon adropion</i> 311				0.1	0.1
<i>Diphascon cf. dastychi</i>	0.3	<0.1	<0.1		0.3
<i>Diphascon cf. victoriae</i>				0.3	0.3
<i>Hypsibius</i> sp.	0.3	<0.1			0.3
<i>Hypsibius</i> sp. 200				0.1	0.1
<i>Hypsibius cf. valentinae</i>	0.3	<0.1	<0.1		0.3
<i>Isohypsibius sattleri-bakonyiensis</i>			<0.1	<0.1	<0.1
<i>Macrobiotus cf. pseudohufelandi</i>	0.3	<0.1			0.3
<i>Macrobiotus areolatus</i> group	0.3	0.1			0.4
<i>Macrobiotus hufelandi</i> group		0.4			0.4
<i>Macrobiotus simplex</i>		<0.1			<0.1
<i>Macrobiotus</i> sp. nov.		<0.1	0.3		0.3
<i>Milnesium brachyungue</i>				0.1	0.1
<i>Milnesium</i> sp. nov		<0.1	0.3		0.3
<i>Minibiotus constellatus</i>		0.1			0.1
<i>Minibiotus eichorni</i>				0.3	0.3
<i>Minibiotus</i> sp.	0.3	0.1	0.2		0.6
<i>Minibiotus sidereus</i>				0.3	0.3
<i>Minibiotus simplex</i>		<0.1			<0.1
<i>Murrayon</i> sp.	0.7	0.3	0.3		1.3
<i>Platicrista ramsayi</i>	0.3			0.3	0.6
Mean number of individuals per sample	0.4	0.2	0.2	0.2	0.3

Figure 3.3. MDS ordination of the tardigrade assemblage composition by sample, across all microhabitats sampled. Samples closer together were more similar in composition.

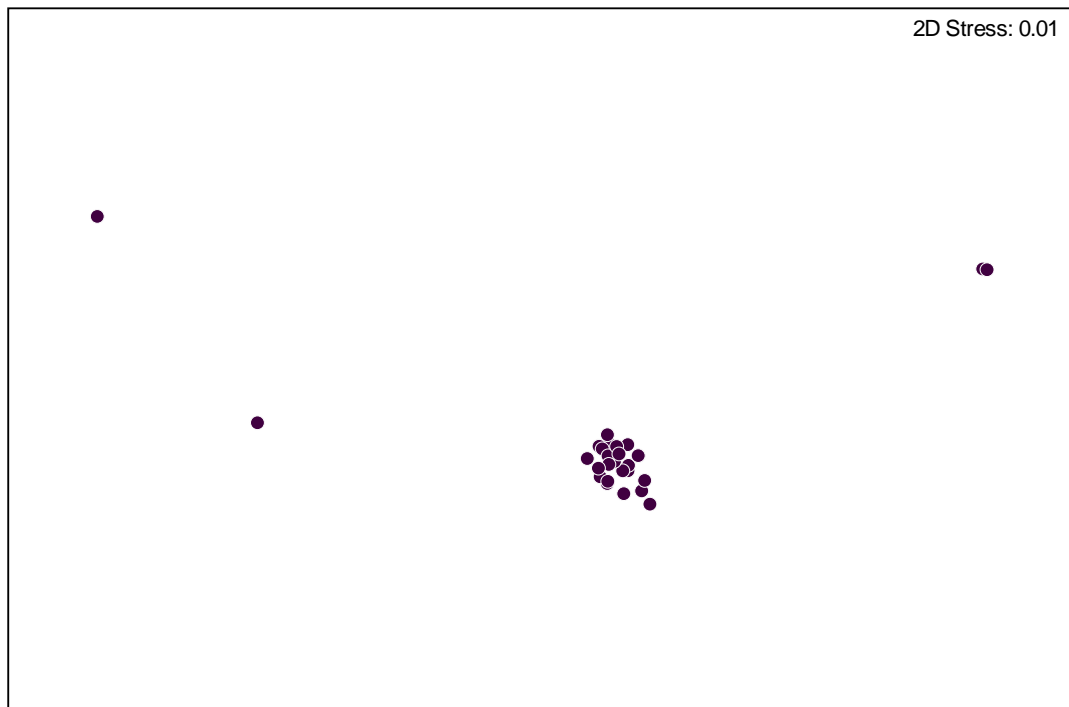


Figure 3.4. MDS ordination of tardigrade taxa found in samples from a *Polylepis* forest and surrounding grassland. Similarities in distributions of taxa among samples.

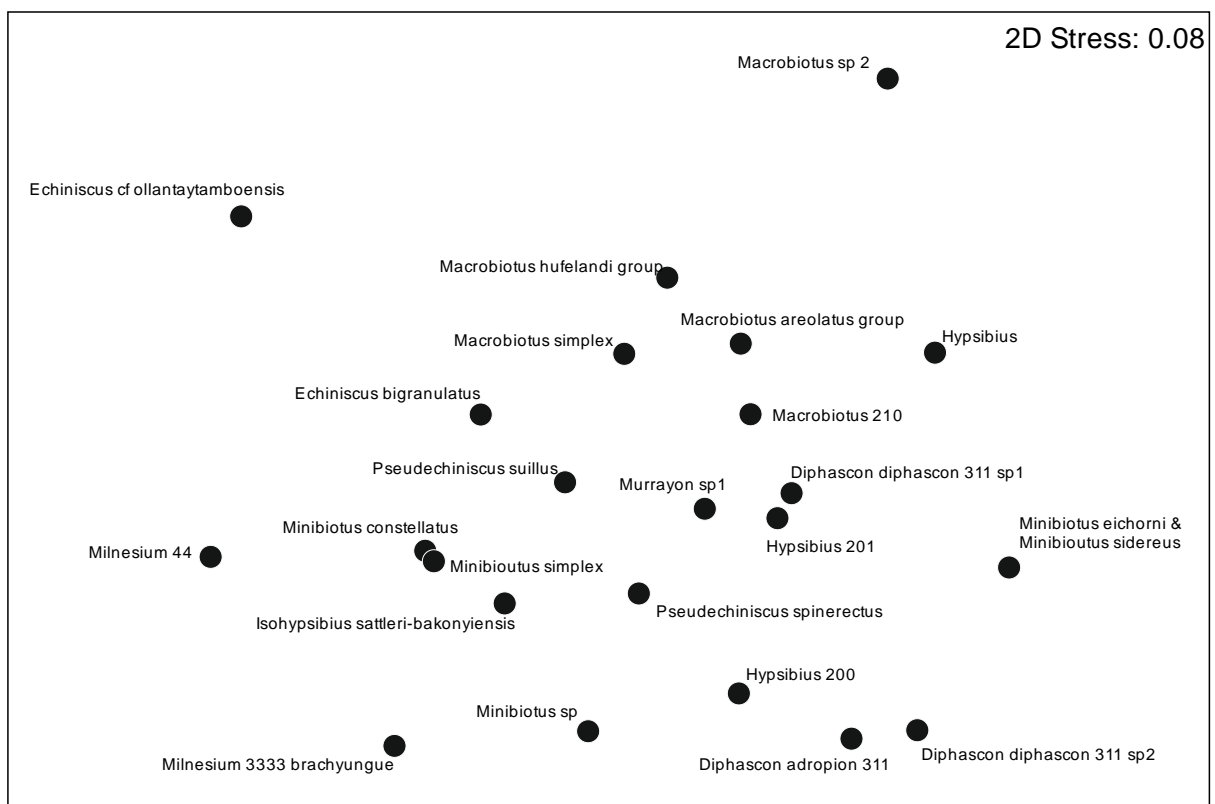


Figure 3.5. MDS ordination of the tardigrade assemblage composition by sample, classified according to forest position: ground (●), trunk (●) and branch (○). MDS 2D stress = 0.07.

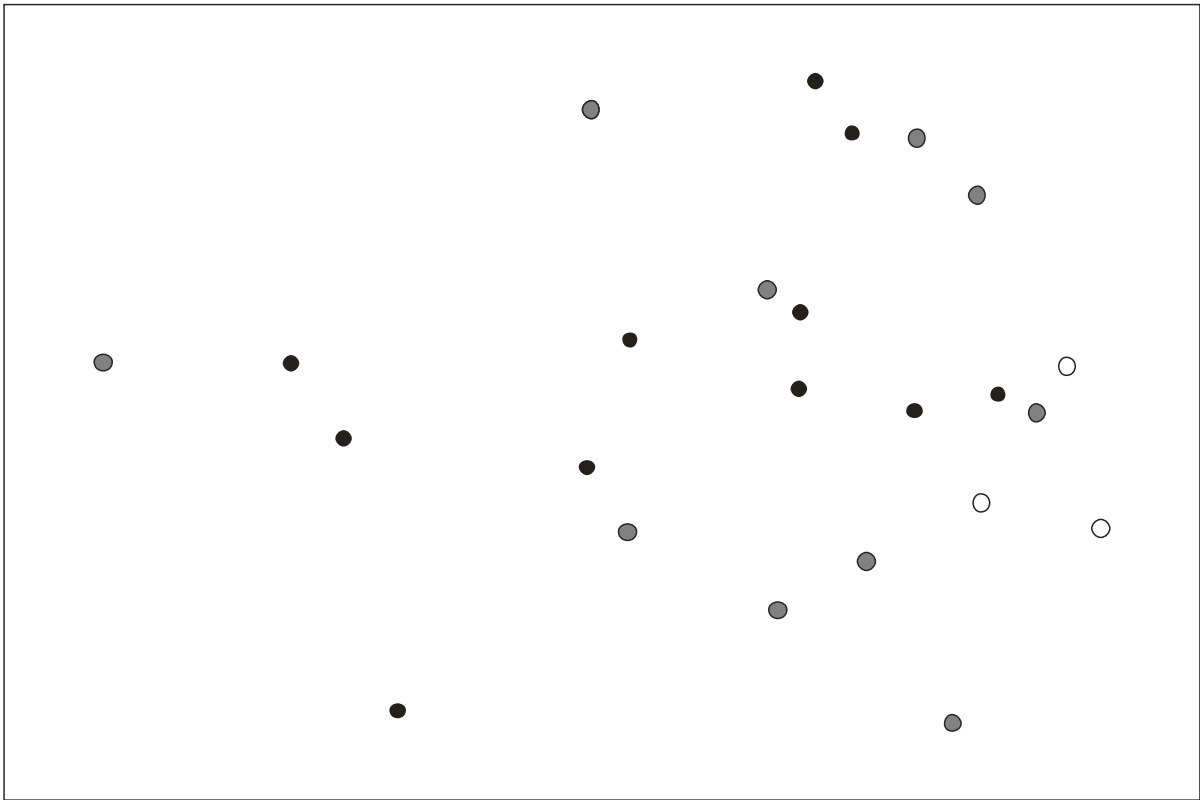


Figure 3.6. MDS ordination of the tardigrade assemblage composition by sample, classified according to host: bryophyte (●), lichen (●), *Polylepis* bark (○). MDS stress = 0.07.

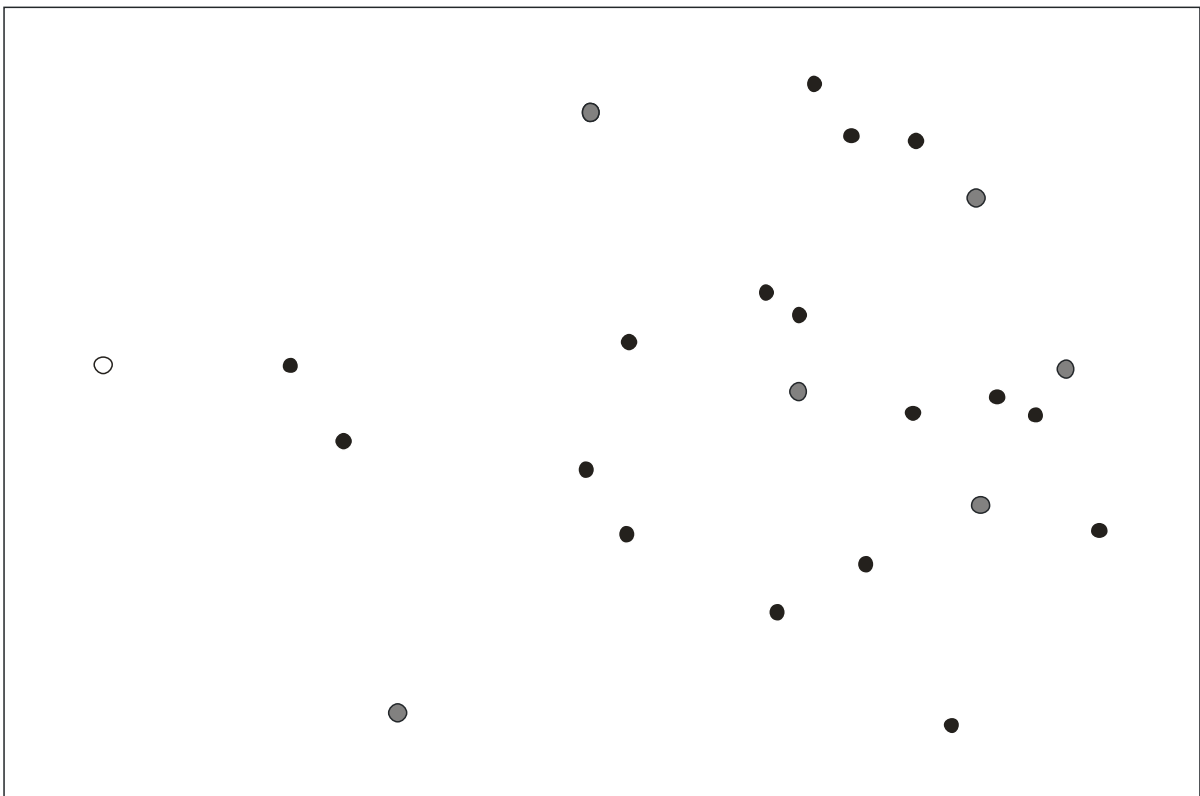
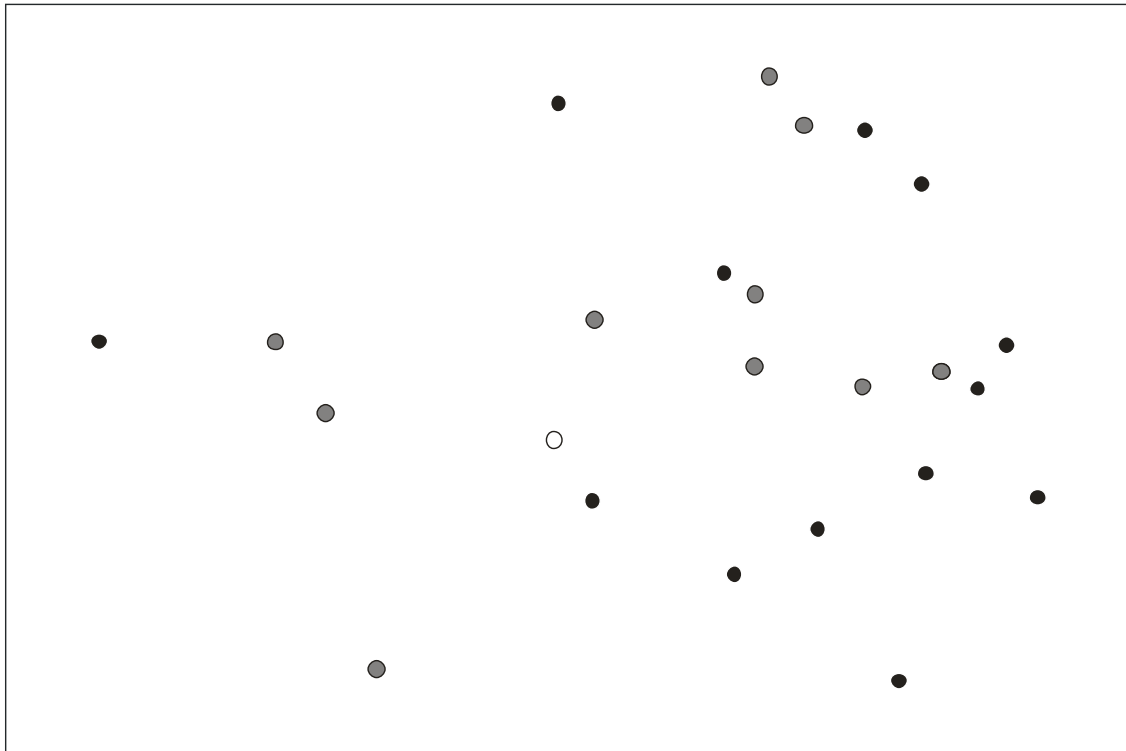


Figure 3.7. MDS ordination of the tardigrade assemblage composition by sample, classified according to substrate: tree (●), rock (●), and soil (○). MDS stress = 0.07.



Discussion

This study represents the first attempt to document the tardigrade composition of high altitude Andean *Polylepis* woodlands and associated grasslands in detail. Indeed, there are few publications on tardigrades for most of South America. Published work up to date has reported just 28 tardigrade taxa for Peru (Kazmareck *et al.*, 2014), illustrating the poor state of knowledge of tardigrade diversity in this region. Our study has added two more genera to those recorded for Peru (*Calcarobiotus* and *Diphascion*) and three confirmed new species belonging to the genera *Calcarobiotus*, *Macrobiotus* and *Milnesium*, with potentially more to come after additional review of existing species descriptions.

The tardigrades found in this study belong to two classes: Eutardigrada and Heterotardigrada. Eutardigrades were highest in OTU richness while heterotardigrades presented lower richness. This corresponds to patterns found in quantitative studies in Spain (GuilSanchez-Moreno *et al.*, 2009, Guil and Sanchez-Moreno, 2013). Higher eutardigrade diversity is often associated with humid environments, while heterotardigrades are typically most diverse in drier conditions (Bertolani *et al.*, 1987, Ito, 1993, Ito, 1999, GuilSanchez-Moreno *et al.*, 2009). However, some other studies

have found higher abundance of heterotardigrades than eutardigrades (GuilHortal *et al.*, 2009, GuilSanchez-Moreno *et al.*, 2009, Guil and Sanchez-Moreno, 2013), though their relative abundances can vary greatly from sample to sample (Maucci, 1980, Kathman and Cross, 1991, Degma *et al.*, 2017, Glime, 2017). *Polylepis* forests are more humid environments than the surrounding grasslands, which might favour higher numbers of eutardigrades. The few grassland samples in our study resulted in the discovery of only a single tardigrade taxon in this environment (*Calcarobiotus* sp. nov.), preventing more detailed comparisons with the forest samples.

Five taxa from the class Heterotardigrada were found in our study, two from the genus *Echiniscus* and three from *Pseudechiniscus*, all of which were more abundant in moss from *Polylepis* branches than elsewhere. This fits with the supposed preference of these taxa for drier conditions (Bertolani *et al.*, 1987, Ito, 1993, Ito, 1999), since the environment associated with branches is much drier than that of tree trunks and the forest floor. The relative size of host material in these locations—small hosts on small twigs in the canopy, contrasting with larger patches of hosts on the floor—makes the host environment in the tree canopy more prone to desiccation than that closer to the ground. The more humid environment on the forest floor helps to explain the dominance of Eutardigrada in those samples.

Tardigrade composition was significantly different between samples from the ground and samples from branches. This is partly explained by the differences at Class level explained above, but even within the Eutardigrada, several taxa were more abundant in branch samples, such as *Minibiotus eichhorni*, *Minibiotus sidereus*, and *Hypsibius* 200. Nine taxa were only found in samples from the forest floor (e.g., *Macrobiotus hufelandi* group). In fact, tardigrade taxa did not show similar occupancy patterns among the samples: each taxon tended to have a unique (potentially idiosyncratic) distribution which was not shared closely by any other taxon. This variability from sample to sample on the ground helps to explain the significantly different dispersion in the ordination compared with the more consistent branch samples.

The wide range of humidity within the forest structure, from the more humid, shaded forest floor to the drier, sunnier canopy, might promote a greater overall diversity of tardigrades within the forest, in the manner suggested by Bertolani and Rebecchi (1996). In this study, for the first time, bark was analysed as a potential tardigrade

habitat. The papery bark of the *Polylepis* trees provides habitat for a species of at least one genus (*Macrobiotus* sp. nov.).

Tardigrade composition did not differ significantly between hosts (bryophytes, lichens, bark) or substrates (rock, soil, tree). Only a few other studies have attempted quantitative comparisons of tardigrades in hosts and substrates (Guil Sanchez-Moreno *et al.*, 2009, Kaczmarek Goldyn Welnicz *et al.*, 2011, Guil and Sanchez-Moreno, 2013). Such studies are hampered by the great variation in abundance from sample to sample. For example, Kaczmarek Goldyn Welnicz *et al.* (2011) found slightly higher tardigrade abundance and diversity in bryophytes than in lichens, but they argued that this was probably the result of uneven sampling.

The current study demonstrated a high number of empty samples (59%) and, of the 29 samples which contained tardigrades, 11 held just a single individual. Tardigrade abundance was not affected by sample weight. Statistical comparisons from such datasets are difficult to interpret. For example, the lack of significant differences between hosts and substrates might result from the limited number of occupied samples available for comparison, rather than from indifference by tardigrades. For more conclusive quantitative studies of tardigrade associations with hosts and substrates, including ones in *Polylepis* forests, much greater replication of larger samples would be needed.

More general observations on host preferences by tardigrades have been made, but the conclusions have been mixed. Hofmann (1987) and Dastych (1987) both reported an association between bryophyte species and tardigrade taxa. However, Ramazzotti and Maucci (1983) and Kathman and Cross (1991) found no relationship between bryophyte species and tardigrade distribution.

As found here, a common feature of sampling tardigrades is their potentially high variability in abundance at fine spatial scales, which can result in patchy datasets with many samples containing few or no organisms. In order to overcome this problem, future studies should consider much higher levels of sample replication. In studies comparing tardigrade distribution patterns among several locations, hosts or substrates, such replication will make significant demands for processing and identifying the samples. Nevertheless, such studies would extend considerably the

understanding of tardigrade (and potentially other micrometazoan) distribution patterns at fine scales.

Before comparing tardigrade distribution patterns at broader scales, it is essential to understand the fine-scale variation, particularly in terms of the way it might affect sampling outcomes and interpretations. For example, the current study has demonstrated that tardigrade composition differs according to location (forest floor versus tree canopy) and so comparisons between forests or through time must design their sampling strategies carefully (and document them clearly) in order that reliable assessments can be made.

On the evidence of this study, many tardigrades seem able to live in several hosts and substrates, while others might be restricted to more specialist habitats. Exploring these associated patterns and mechanisms would help the understanding of micrometazoan ecology more generally.

4

The structure of tardigrade communities at the landscape scale: the influence of habitat and host

Introduction

Even though microorganisms comprise much of Earth's biodiversity and play crucial roles in ecosystem functioning, little is known about their distribution patterns at different scales, compared with our understanding for plants and larger animals (Green *et al.*, 2004, Green and Bohannan, 2006). Their biogeography had been considered of little concern because of the assumption microscopic organisms were not limited by biogeographical barriers and distances, but this assumption has more recently been challenged (Fontaneto *et al.*, 2006).

There are now two principal hypotheses concerning distribution patterns in micrometazoans: the cosmopolitan model and the moderate endemism model. The cosmopolitan model assumes "everything is everywhere but the environment selects" (Bass-Becking, 1934) which implies that microscopic organisms (less than 2 mm) with high dispersal rates have cosmopolitan distributions. The local environmental conditions determine which taxa survive (Fenchel *et al.*, 1997, Finlay, 2002, Fenchel and Finlay, 2004). On the other hand, the moderate endemism model suggests that some microscopic taxa have restricted distributions while others are cosmopolitan. The local environmental conditions still determine which taxa survive from those available in the regional species pool (Foissner, 1999, Foissner, 2006, Foissner, 2008). At present, it is difficult to determine which model is more appropriate because there is limited information about distribution patterns of microscopic organisms (Lachance, 2004, Fontaneto *et al.*, 2006).

Regardless, at the local level both models consider that local environmental conditions determine which taxa survive. However, for microscopic organisms, it is not clear how "environment" should be defined. At the microscopic scale, environment can be seen as a nested series of interacting local conditions (e.g., the environment provided by a bryophyte host inside the environment determined by understorey vegetation and immediate canopy conditions inside the environment of a forest). The relative

importance of these nested environments on micrometazoan distribution and abundance is not well understood.

Tardigrades provide a good model for looking at micrometazoan distribution patterns but their ecology and distribution patterns has been little studied (Guil and Giribet, 2012, Guil and Sanchez-Moreno, 2013). Tardigrades are relatively abundant in a wide range of environments, from pole to pole (Marcus, 1928, Czechowski *et al.*, 2012, Guil and Sanchez-Moreno, 2013). Like other micrometazoans, tardigrades can enter dormancy, leading to a high survival rate and the ability to survive extreme conditions (Jönsson *et al.*, 2005, Horikawa *et al.*, 2006a, HorikawaKunieda *et al.*, 2008, Jönsson *et al.*, 2008, Rebecchi *et al.*, 2009).

Some tardigrades live in bryophyte hosts and each host might offer different environmental conditions and resources (Guil and Sanchez-Moreno, 2013), though little is known about tardigrade habitat associations (GuilSanchez-Moreno *et al.*, 2009). It is commonly assumed that species-specific habitat patterns do exist in these animals (Ito, 1991, Ito, 1993, Ito, 1995, Bertolani and Rebecchi, 1996, Ito, 1997, Guidetti *et al.*, 1999, Ito, 1999, Guidetti and Bertolani, 2001). Some more generalist taxa have broader niche requirements, allowing them to live in several hosts (Degma, 2003, Degma and Pecalkova, 2003, Degma, 2006), while other tardigrades taxa are more specialist, restricted to certain host species (Bertolani and Kinchin, 1993).

However, bryophyte hosts also live within habitats of their own (e.g., forest or grassland), which modify the environment and resources more generally. At the landscape scale, different species of bryophytes live in habitats such as bog, grassland and forest, and each of these habitats might offer different environmental conditions for tardigrades even if the host is the same (Glime, 2017). Some bryophyte hosts have broad niche requirements which allow them to live in a variety of habitats, but others tend to be present in specific habitats (Frahm, 2009, Granzow-de la Cerda *et al.*, 2016). Diverse habitats such as bog, grassland and forest offer different environmental conditions for tardigrades (Richardson, 1999, Richardson, 2000, Richardson *et al.*, 2005). For example, closed forest may offer more stable conditions for particular tardigrade assemblages (Richardson *et al.*, 2005), while other habitats with drier conditions have more extreme conditions, and present higher instability and low levels

of humidity (Guil and Sanchez-Moreno, 2013). These differing conditions are also likely to influence the occupancy and abundance of other species which might interact with tardigrades (Holz *et al.*, 2002, Adams *et al.*, 2014, Bielańska-Grajner *et al.*, 2017).

How the environmental conditions and resources at the bryophyte host level interact with those at the habitat level will strongly influence tardigrade composition. Yet it is not clear whether tardigrade taxa are associated more with specific bryophyte hosts or with specific habitats regardless of host. The relative abundance of generalists and specialists for bryophyte hosts and habitats is also poorly documented.

This study aims to compare tardigrades in bryophyte hosts in three different environments (bog, forest and grassland) in an Andean mountain landscape. The study also considers the potential interaction between habitat and host scales in determining tardigrade community structure.

Methods

Bryophyte samples were collected from three different habitats—bog, forest and grassland—in El Ángel Ecological Reserve, Carchi Province in northern Ecuador. The bog samples were collected from a permanently wet ombrotrophic (rain-fed) bog dominated by *Oreobolus* cushion plants, but without pools of water (slope 0%, altitude 3676 m, UTM 18 N 180585 74994). The forest samples were collected from the floor of a *Polylepis sericea* forest (slope 0%, altitude 3575 m, UTM 18 N 168316 78347), with grazing by livestock and occasional visits by tourists. Bryophyte samples from the páramo grassland were collected from an area next to the *Polylepis* forest (slope 35%, altitude 3575 m, UTM 18 N 168316 78347). The vegetation of this grassland was dominated by *Calamagrostis* tussock grasses and *Espeletia* giant rosette plants.

Nine bryophyte samples were collected from each of the three habitats: three replicates of each of three bryophyte host species. *Leptodontium longicaule* Mitt. was collected from all three habitats. *Breutelia* sp. and *Campylopus* sp. were collected from both grassland and bog habitats, but were not present in the forest. Instead, the two commonest forest floor bryophytes were sampled: *Thuidium delicatulum* (Hedw.) Schimp and *Pleurozium schreberi* (Brid.) Mitt.

Replicate samples of approximately 4 cm³ uncompressed volume were collected from pure monospecific patches of the host bryophyte species. Samples were air-dried in individual paper envelopes, and stored at 10–25 °C until tardigrades were extracted.

In the laboratory, dried samples were rehydrated in water for 16–24 h. Rehydrated samples were shaken and passed through stacked sieves of 180 µm and 38 µm mesh. Material retained by the small aperture mesh sieve was searched for tardigrades using a Kyowa SDZ-PL stereoscopic microscope with 30–40x objectives (Kyowa, Japan).

Tardigrades were mounted individually on microscope slides under cover slips in Hoyer's mounting medium. The identification of tardigrades was done to Operational Taxonomic Units (OTUs) with a Leica DMLB microscope with 40x and 100x objectives (the latter with immersion oil), using Guidetti and Bertolani (2005), Marley *et al.* (2011), and Degma *et al.* (2017).

Overall tardigrade numbers, OTU richness and Shannon diversity of samples were analysed using one-way General Linear Model (GLM ANOVA), after confirming normality with a Shapiro-Wilks Test. These statistical tests were carried out with R version 3.3.3 (R Core Team, 2017).

The OTU composition of samples was compared using non-metric Multidimensional Scaling (MDS) in Primer 6 (Primer-e, Plymouth, UK), on square-root transformed OTU count data. The graphical output of this approach positions samples with similar composition close together and samples with more different composition further apart. Statistical differences in sample composition were determined by permutational ANOVA (PERMANOVA) using the PERMANOVA+ add-on to Primer 6. In this exploratory study, with low levels of replication, a *p*-value of 0.1 or less was considered sufficient to merit consideration of a difference, with caution. PERMANOVA is sensitive to differences in the dispersion of data (Anderson *et al.*, 2008) and so an additional test, when significant differences were identified by PERMANOVA, was carried out to identify any significant differences in dispersion between groups, using PRIMER's PERMDISP.

Results

Across all twenty-seven samples (bog, forest and grassland), 46 tardigrade OTUs were identified from 538 individuals (Fig. 4.1). Some tardigrades found in this study represent new taxa: *Hypsibius* sp. nov. 200, *Hypsibius* sp. nov. 201, *Isohypsibius*

saulrogersi sp. nov. and *Isohypsibius* sp.1 210, which will be described separately. *Macrobiotus* was the most abundant genus, and the only one present in all bryophyte species and in all habitats examined (Fig. 4.1). Some other tardigrade genera were also abundant in the samples: *Adropion*, *Paramacrobiotus*, *Diphascon* and *Hypsibius* (Fig. 4.2). In contrast, other OTUs were rarely observed in this study, occurring as single individual records: e.g., *Adropion* cf. *scoticum*, *Mesocrista* sp., *Mixibius* sp. 210, and *Ramazzottius* sp. (Fig. 4.1).

Twenty tardigrade OTUs, comprising 71 specimens, were found in the bog samples (Fig. 4.1). Individual samples contained 1–33 individuals and up to 12 OTUs. Eutardigrades outnumbered heterotardigrades in abundance (66 vs 5 individuals) and taxon richness (18 vs 2 taxa). Thirty-two OTUs were found across 421 specimens in the forest host samples (Fig. 4.1). Individual samples here contained 27–74 individuals and up to 15 OTUs. Eutardigrades again outnumbered heterotardigrades in abundance (401 vs 20 individuals) and taxon richness (32 vs 1 taxa). Eleven OTUs were found across 46 specimens in the grassland host samples (Fig. 4.1). Individual samples here contained 1–20 individuals and up to 8 OTUs. Only eutardigrades were found in the grassland samples.

Thirty-three tardigrade OTUs (72%) were found in only one of the three habitats: 20 in the forest, 12 in the bog, and one in the grassland (Figure 4.1). Nine OTUs (20%) were present in samples from two habitats: five in forest and grassland, three in bog and forest, and one in bog and grassland. Four OTUs (9%) were present in samples from all three habitats.

Twenty-seven tardigrade OTUs (59%) were found in only one of the five host: one in *Breutelia*, one in *Campylopus*, 15 in *Leptodontium*, 8 in *Pleurozium* and 2 in *Thuidium*. Six tardigrade OTUs (13 %) were found in two of the hosts, eight (17%) in three hosts, four (9%) in four hosts, and just one (2%) was present in samples from all five hosts (Figure 4.1).

For all hosts combined, forest samples had higher tardigrade abundances, OTU richness and diversity indices than grassland and bog samples (respectively: ANOVA $F_{2,24} = 28.81, p < 0.001$; ANOVA $F_{2,24} = 17.60, p < 0.001$; ANOVA $F_{2,24} = 10.23, p = 0.001$; Table 4.1). The bog and grassland samples were not significantly different in all three cases. Forest samples also had higher tardigrade abundances than the two other habitats for

Leptodontium, the only host found in all three habitats (ANOVA $F_{2,6} = 7.56$, $p = 0.023$). OTU richness and diversity indices for the *Leptodontium* host were not significantly different in any of the three habitats (ANOVA $F_{2,6} = 3.35$, $p = 0.106$; ANOVA $F_{2,6} = 2.56$, $p = 0.157$; Table 4.1) though mean values followed a similar pattern to that found for all hosts combined. In the forest samples, *Pleurozium* and *Thuidium* hosts had similar levels of abundance and diversity to *Leptodontium* (ANOVA for N $F_{2,6} = 3.42$, $p = 0.102$; S $F_{2,6} = 2.01$, $p = 0.215$; H' $F_{2,6} = 1.03$, $p = 0.412$; Fig. 4.3A–C). Both *Campylopus* and *Breutelia* hosts were not significantly different in tardigrade abundance and diversity, though one-third of the samples were empty.

Bog and grassland samples were more variable in composition than forest samples (Figs 4.4 & 4.5A). Although the centroids of forest host samples were closer together in the MDS plot than those of the two other habitats, all forest hosts were different (PERMANOVA $p \leq 0.1$, PERMDISP $p = 0.607$) but hosts in bog and grassland were too variable to be separated in almost every case ($p > 0.1$). The similarity in tardigrade composition between habitats in all three comparisons was 14.9–16.6% (Fig. 4.5A).

For *Leptodontium* samples only, each habitat was more consistent in composition than across all the hosts (Figs 4.5B & 4.6). All habitats were different for the *Leptodontium* host (PERMANOVA $p \leq 0.1$, PERMDISP $p = 0.673$). Grassland samples were as similar in tardigrade composition to forest samples as they were to each other (both cases 40.9%), but lower when compared with bog samples (26.7%), while bog and forest samples were only 14.9% similar (Fig. 4.5B).

Several tardigrade OTUs were present in many of the samples and are clustered together in the centre of Fig. 4.7: for example, *Adropion* sp. 300, *Diphascon* sp. 311, *Hypsibius* sp. 210, *Macrobiotus* sp. 210, *Paramacrobiotus* sp. Rarer, less abundant OTUs were located around the periphery of the figure (such as *Adropion* cf. *scoticum* 311, *Isohypsibius* cf. *brevispinosus*, *Mesobiotus* sp. 301, *Mixibius* sp. 210).

Figure 4.1. Tardigrade OTUs present in bryophyte hosts from three habitats in Carchi Province, Ecuador: a bog at 3676m, a *Polylepis* woodland at 3575 m, and surrounding páramo grassland. A. Sample occupancy of each OTU in three replicate samples. B. Mean abundance of each OTU in the same samples. Coloured circles indicate each habitat: blue for bog, green for forest and red for grassland. Host and habitat pairings are indicated by an abbreviation at the foot of the panel: BL=Bog *Leptodontium*, BB=Bog *Breutelia*, BC=Bog *Campylopus*, FT=Forest *Thuidium*, FP=Forest *Pleurozium*, FL=Forest *Leptodontium*, GL=Grassland *Leptodontium*, GB=Grassland *Breutelia*, GC=Grassland *Campylopus*. OTUs including “cf” and “sp.” followed by a number refer to recognizable morphospecies, some of which are new to science.

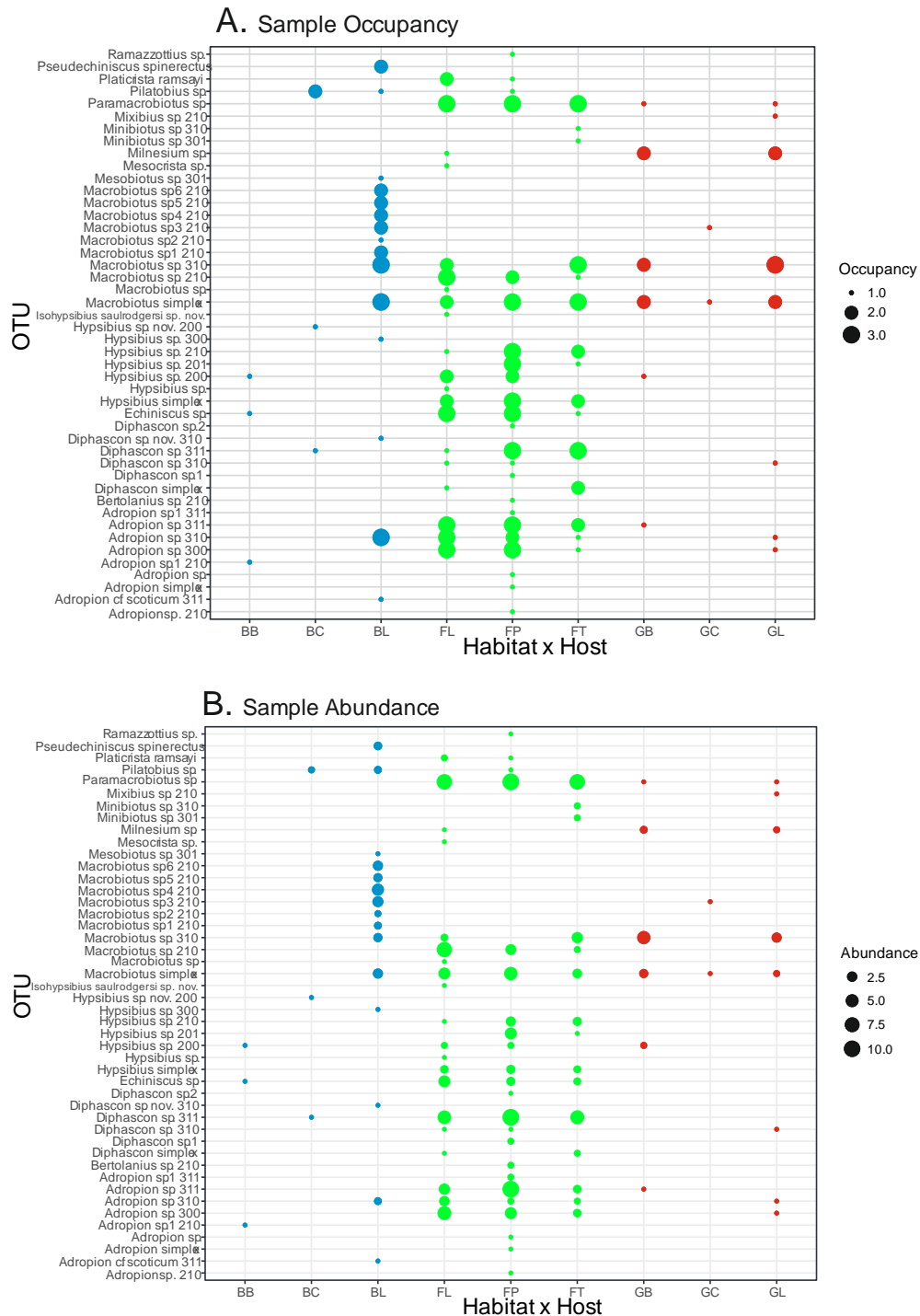


Figure 4.2. The most abundant tardigrade genera across samples combined for all three habitats (bog, forest and grassland).

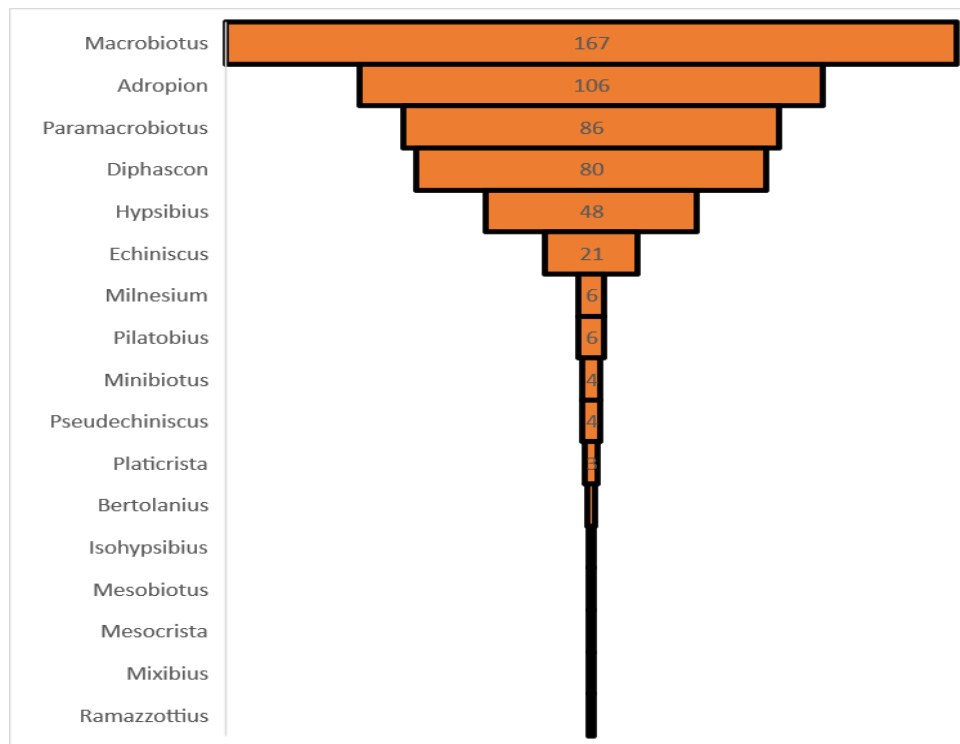
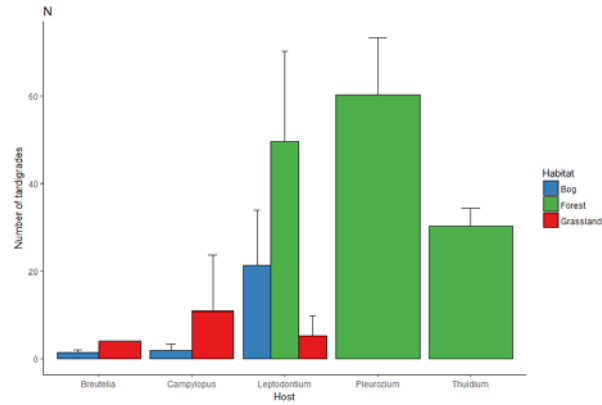


Table 4.1. Number of tardigrades (N), number of OTUs (S) and Shannon Diversity Index (H') in each habitat type for all hosts combined and for *Leptodontium* only. Means \pm sd are given. Means sharing a letter within a column were not significantly different.

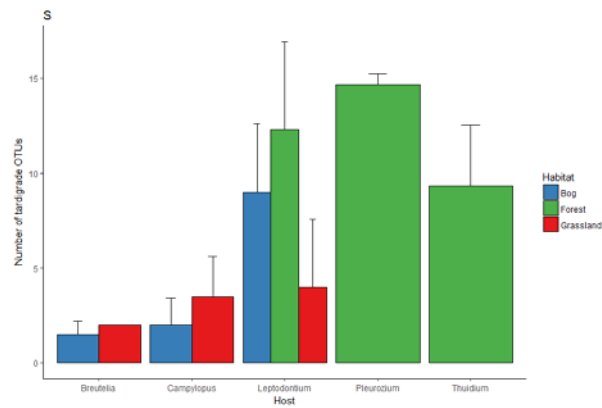
Host	Sample n	N	Overall S	S	H'
All hosts combined					
Forest	9	46.8 ^a \pm 18.0	33	12.0 ^a \pm 3.8	2.0 ^a \pm 0.3
Bog	9	7.9 ^b \pm 11.9	20	3.8 ^b \pm 4.4	0.8 ^b \pm 0.9
Grassland	9	5.1 ^b \pm 6.4	12	2.6 ^b \pm 2.6	0.7 ^b \pm 0.6
Grand Total	27	19.9 \pm 23.1	47	6.1 \pm 5.5	1.2 \pm 0.9
<i>Leptodontium</i> only					
Forest	3	49.7 ^a \pm 20.5	20	12.3 ^a \pm 4.6	2.1 ^a \pm 0.3
Bog	3	21.3 ^b \pm 12.6	15	9.0 ^a \pm 3.6	1.9 ^a \pm 0.4
Grassland	3	5.3 ^b \pm 4.5	8	4.0 ^a \pm 3.6	1.0 ^a \pm 1.0
Grand Total	9	25.4 \pm 23.0	33	8.4 \pm 5.0	1.7 \pm 0.8

Figure 4.3. Abundance and diversity descriptors for bryophyte hosts in three habitats. **A.** Mean number of tardigrade OTUs. **B.** Mean species richness. **C.** Mean Shannon diversity index. Coloured bars indicate each habitat: blue for bog, green for forest and red for grassland. Error bars represent one standard deviation.

A. Species richness



B. Number of tardigrade OTUs



C. Shannon Diversity Index

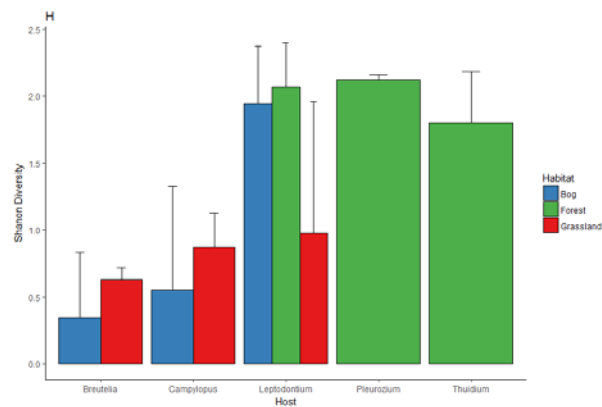


Figure 4.4. MDS ordination of tardigrade OTU composition for five bryophyte hosts in three habitats. Samples located close together in the figure had similar compositions of tardigrades, whereas those further apart were more different in composition. Coloured symbols indicate each habitat: blue for bog, green for forest and red for grassland. Host and habitat pairings: BL=Bog *Leptodontium*, BB=Bog *Breutelia*, BC=Bog *Campylopus*, FT=Forest *Thuidium*, FP=Forest *Pleurozium*, FL=Forest *Leptodontium*, GL=Grassland *Leptodontium*, GB=Grassland *Breutelia*, GC=Grassland *Campylopus*.

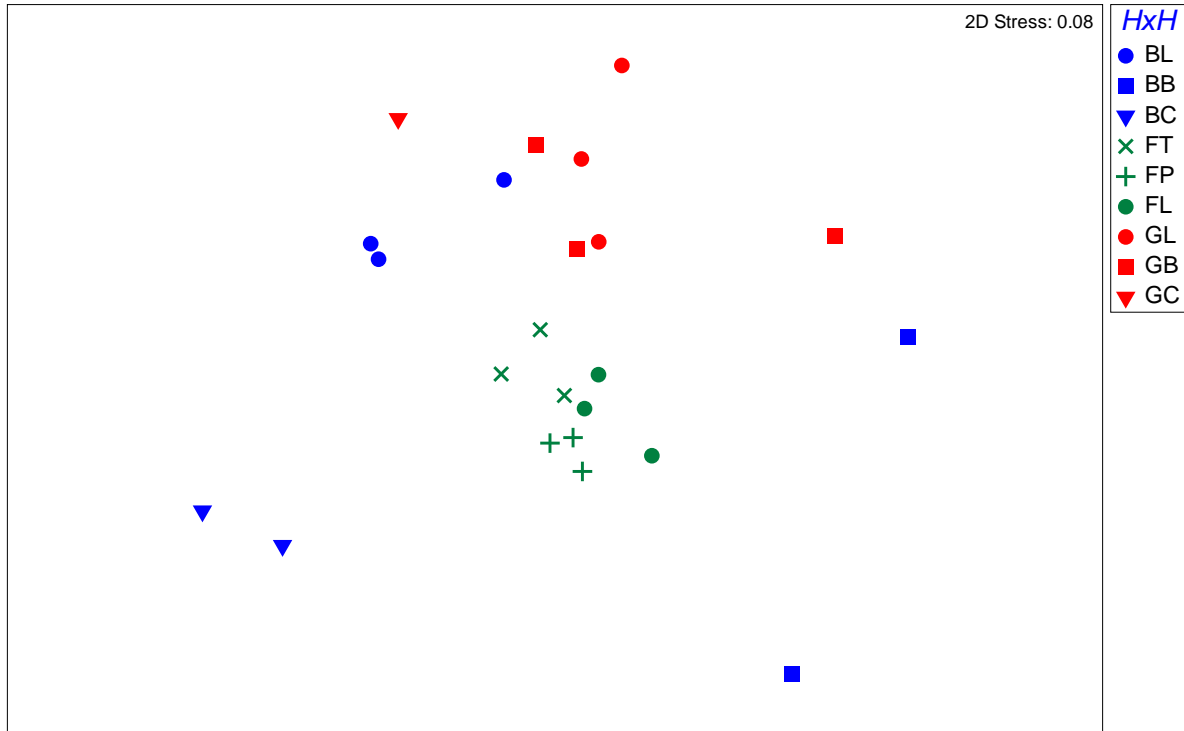


Figure 4.5. Similarity in tardigrade OTU composition between and within three habitat types for **A.** all bryophyte hosts combined and **B.** for the *Leptodontium* host only. The circles represent the mean percentage similarity of samples within each habitat. The connections between circles show the mean percentage similarity of samples between the habitats.

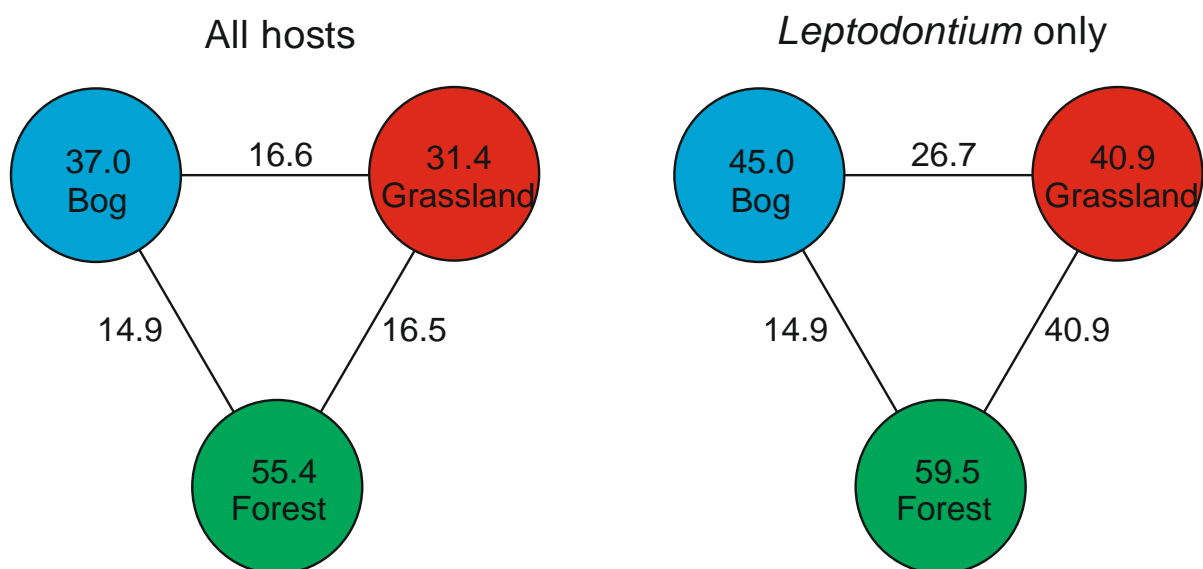


Figure 4.6. MDS ordination of tardigrade OTU composition for the *Leptodontium* host in three habitats. Coloured symbols indicate each habitat: blue for bog, green for forest and red for grassland.

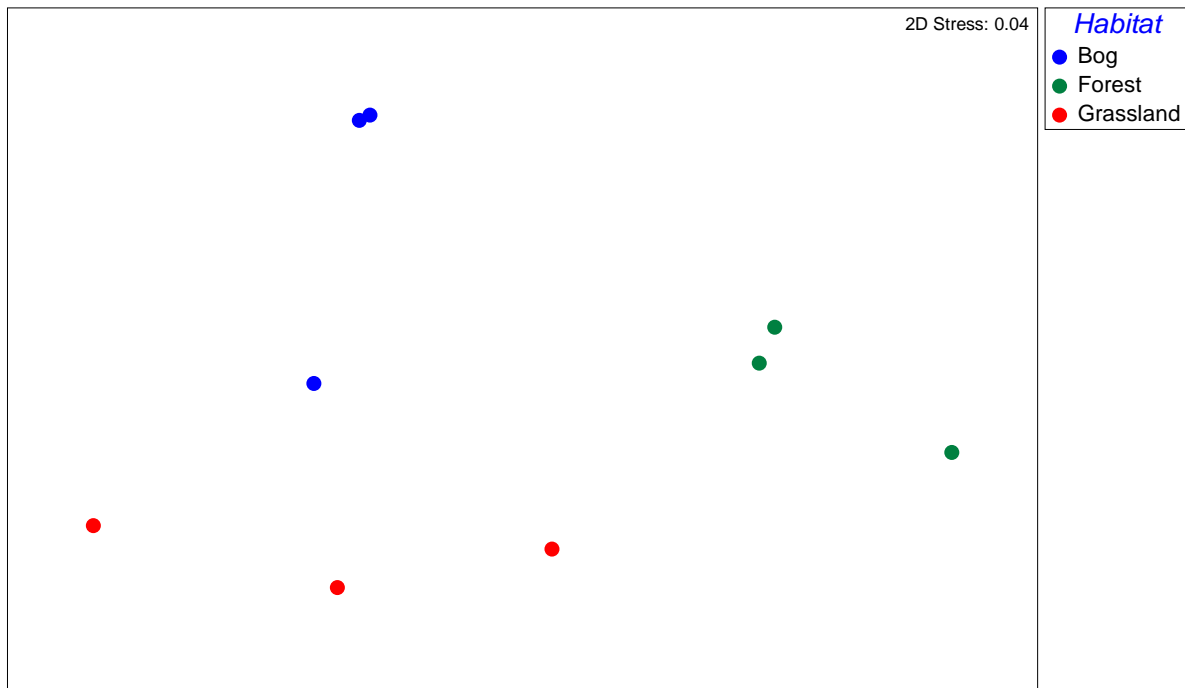
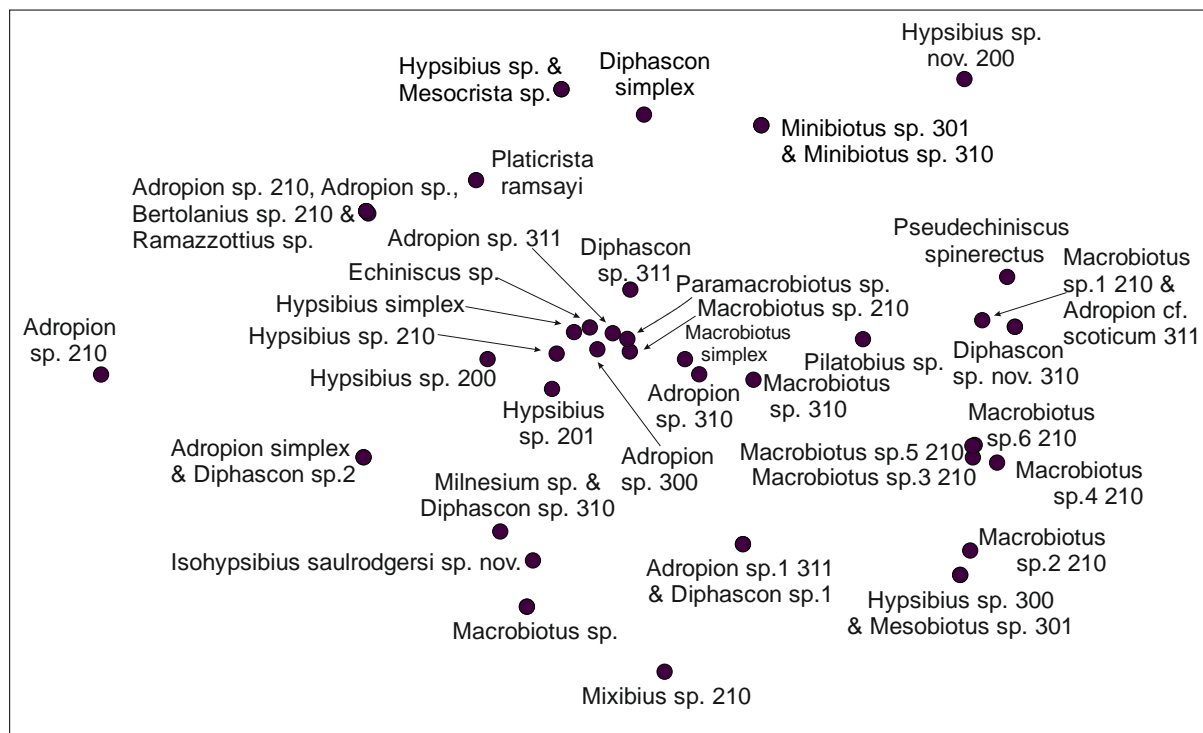


Figure 4.7. MDS ordination of tardigrade OTUs within the samples. OTUs located close together in the figure tended to be found in similar samples, whereas OTUs far apart in the ordination were found in different samples. In several cases, more than one OTU shares the same symbol. 2D Stress = 0.08.



Discussion

It is challenging and time consuming to identify tardigrade taxa to generic or species level, because determination of species requires the review of many widely dispersed descriptions and requires a certain amount of technical and linguistic expertise (Krell, 2004). In addition, several new species were found in this relatively limited study. Unfortunately, ecological differences at the generic level or above have not been sufficiently documented to permit comparisons using those broader taxonomic categories.

However, ecological studies are possible, even if species cannot be named. In this study, OTUs provided a relatively rapid yet effective way of comparing samples—an approach that has been used widely for other “difficult” animal groups in ecological studies (Hackman *et al.*, 2017), e.g., the use of morphospecies in micrometazoans such as nematodes (Traunspurger *et al.*, 2017). However, the use of such taxonomic units should be treated with caution to avoid overestimation of species number (Krell, 2004). More recently, the use of molecular OTUs (MOTUs) for tardigrade studies has been developed (Czechowski *et al.*, 2012), but it is not yet sufficiently advanced to be used widely in studies such as the one described here.

It has been reported from the few other quantitative studies of tardigrade abundance and occupancy that the resulting data are very variable (GuilSanchez-Moreno *et al.*, 2009, KaczmarekGołdyn *et al.*, 2011). This makes it rather difficult to compare tardigrade diversity among samples and studies with quantitative data (Schuster and Greven, 2007). It is not clear why the variability is so high, which itself suggests a lack of understanding of tardigrade ecology. This study follows the same pattern, with some taxa more common while others were rare. Four samples (15% of those taken) had no tardigrades at all.

Macrobiotus species were found across a range of host and habitats. This agrees with findings from other studies from other continents, where this genus was the most abundant inhabitant of host bryophytes (Grabowski, 1995, McInnes *et al.*, 2001, Schuster and Greven, 2007, Glime, 2017). On the other hand, some taxa in this study appeared as potential specialists, e.g., *Mixibius* sp. in the grassland, *Ramazzottius* sp. in the forest and *Pseudechiniscus spinerectus* in the bog. However, rare generalist species could give the appearance of being restricted, if they appear once in a small number of

samples. It would be prudent to consider these taxa as *potentially* restricted until further sampling provides more convincing evidence.

Dispersal abilities determine the present and potential distribution range of species but little is known about mechanisms and ranges involved in different dispersal modes at small spatial scales (Thomas and Lana, 2011). For tardigrades, little has been documented about modes of dispersal, and their impact on distribution patterns remains speculative. Trophic groups and population dynamics also likely to be relevant to the abundance and diversity of tardigrades but, again, information is scarce (Traunspurger, 2002). These deficiencies illustrate the need for more quantitative studies of tardigrade abundance and diversity, to establish the patterns, before a effective discussion of mechanisms can take place.

59% of OTUs were restricted to just one host (e.g., *Isohypsibius saulrodgersi* sp. nov., *Mixibius* sp 210, *Ramazzottius* sp.), and 89% of tardigrade OTUs were restricted to three or fewer hosts e.g. *Adropion* sp. 300 and *Pilatobius* sp. Some studies have reported associations between hosts and tardigrades (Hallas, 1978, Hofmann, 1987, Grabowski, 1995, Degma, 2003, Degma and Pecalkova, 2003, Degma, 2006), and others have suggested that hosts affect tardigrade composition to some degree (Hallas, 1978, Hofmann, 1987, Grabowski, 1995, Degma, 2003, Degma and Pecalkova, 2003, Degma, 2006, Schuster and Greven, 2007). However, other studies have not found any association (Bertolani, 1983, Meyer *et al.*, 2003). Thus, published relationships between tardigrades and their hosts have been contradictory (Glime, 2017), but most of these studies were qualitative and lack good-quality data to support the conclusions made.

Microenvironmental conditions such as temperature insulation, relative humidity and temperature might vary between host bryophytes and might determine the development and maintenance of tardigrade communities—but comparisons of these microclimates have not been carried out.

Structural complexity of the hosts might also offer different conditions for tardigrades (Suzuki, 2003). For example, the structural complexity of some hosts provides protection from extreme conditions (Young and Clifton, 2015) or promotes water retention (Wright, 1991, Schuster and Greven, 2007). In general, mosses are more structurally complex than other bryophytes (Gradstein *et al.*, 2001) and are more likely

to vary in microclimatic conditions. Special arrangements of leaves provide moisture, space for locomotion, foraging and a greater diversity of tardigrades (Schill *et al.*, 2011). Strategies against herbivory vary from host to host, conforming to Howe and Westley's (1988) systems of mechanical protection of the plant's surface (with hairs and spines) and/or chemical protection with toxins that repel or kill herbivores. These defences probably influence the abundance and diversity of tardigrades living in them, especially the presence of chemical compounds (Swain and Hillis, 1959, Liao, 1993, Glime, 2006). Previous studies have reported that *Pleurozium schreberi* has a higher phenolic content than *Thuidium delicatulum* and for certain organisms it was the least preferred of the mosses due to its high phenolic compound (Smith *et al.*, 2001, Glime, 2006). However, despite this, in the study described here *Pleurozium* had higher tardigrade abundance than *Thuidium*. This suggests that chemical compounds are not the only characteristic driving tardigrade occupancy.

Hosts offer different microenvironments and resources not only for tardigrades but also for other organisms. Tardigrade abundance is likely to be affected by interactions with these other organisms, whether positive or negative. Some tardigrade taxa feed on a variety of food sources including rotifers, nematodes, other tardigrades, plant cells, algae, protozoa bacteria and other small invertebrates, e.g., *Milnesium tardigradum* is known to feed on nematodes and rotifers (Marcus, 1928, Kinchin, 1994, Schill *et al.*, 2011). Tardigrades also compete with other organisms for food (Schill *et al.*, 2011, Guil and Sanchez-Moreno, 2013). Thus, the presence of particular tardigrade taxa in a host bryophyte might be the indirect consequence of the presence or absence of other taxa.

Most tardigrades (72% of OTUs) were restricted to a single habitat, most likely owing to the environmental conditions present in those habitats, such as temperature, light, air humidity and soil moisture (Fleeger and Hummon, 1975, Morgan, 1977, Hallas, 1978, Wright, 1991, Grabowski, 1995, Schuster and Greven, 2007). In the landscape in which the current study was carried out, patches of forest and bog sit within a matrix of grassland. The microclimate of forest patches is cooler, temperatures less variable, and air humidity higher than that of the surrounding grassland (Fjelds  and Kessler, 1996, Hertel and Wesche, 2008). Bogs are, by definition, perpetually wet, even during dry spells when the soils of the surrounding grassland become very dry. Furthermore, the grassland is prone to fires during such dry periods, while the forest and bogs are largely

unaffected by these forms of disturbance (Zomer and Ramsay, 2017). These differences between the habitats in this study might form the basis for understanding the restricted distribution patterns of most of the tardigrades encountered.

A few tardigrades occupied more than one habitat: 11% of OTUs occurred in four or five hosts: *Adropion* sp. 311, and *Diphascon* sp. 311, *Echiniscus* sp. *Macrobiotus simplex* and *Paramacrobiotus* sp. For these apparently generalist taxa, their bryophyte hosts might be interchangeable, perhaps because these tardigrades are eurytopic (tolerant of a wide range of environmental conditions), which is supported by the fact that these genera globally do occupy a wide range of environmental conditions (Dastych, 1988, McInnes, 1994).

Resources might also vary between habitats, reflected in protection from extreme conditions (Young and Clifton, 2015), water retention (Schuster and Greven, 2007; Wright, 1991), foraging opportunities (Greven and Schuttler, 2001, Schuster and Greven, 2007), and differences in disturbance regime. The moist, organic soil of Ecuadorian montane forests provided good food resources for tardigrades, rotifers, harpacticoid copepods, as well as other microscopic invertebrates (Dole-Olivier *et al.*, 2000, Ricci and Balsamo, 2000). This is consistent with the observations of the current study that the forest habitat supported higher numbers of tardigrades than the grassland and bog habitats. It is also consistent with a Spanish study that concluded that the humid conditions of dense forest offered an abundance of resources for tardigrades and other micrometazoans (GuilHortal *et al.*, 2009, GuilSanchez-Moreno *et al.*, 2009).

Tardigrade abundance is also likely to be affected by interactions with other organisms and those organisms are also likely to be filtered at the habitat level, and perhaps influence tardigrade numbers through their interaction with them. One controlling mechanism might operate through trophic relationships. The presence of different trophic groups, such as omnivores and predators, in some published studies suggests the existence of complex food webs at this scale (Gange and Brown, 2002, Hohberg and Traunspurger, 2005). Tardigrades can be prey for other organisms such arthropods which feed on a variety of tardigrades (Hyvönen and Persson, 1996). Tardigrades can also be predators. In a study across a range of different habitats, Wright (1991) found an association between one predatory tardigrade and two tardigrade prey species. In the same study, Wright (1991) also suggested competitive exclusion among three

species due to trophic and niche overlap. The structure and functioning of food micrometazoan webs are just beginning to be understood (Traunspurger *et al.*, 2017). It seems likely that tardigrades might compete for resources with other micrometazoans but again, there is little understanding of how such interactions might work, and how important they might be (Yeates *et al.*, 1993, Yeates and Bongers, 1999).

Some tardigrades occupied more than one habitat and might be generalists: 19% of OTUs were found occupying two habitats, and 9% occupied all three habitats. These OTUs were the same taxa which occupied a wider range of host bryophytes (*Adropion* sp. 310, *Hypsibius* sp. 200, *Macrobiotus simplex*, *Adropion* sp. 300, *Diphascon* sp. 310, *Macrobiotus* sp. 210, and *Milnesium* sp.).

Clearly, tardigrade numbers were affected by a combination of host and habitat factors, but habitat was more important. Similarity in tardigrade abundance, diversity and composition was greater among samples from within the same habitats than within the same bryophyte hosts. Within the *Leptodontium* host, habitat differences in tardigrade numbers and composition were still pronounced, though diversity differences were not significant at this level of replication.

Therefore, habitat heterogeneity appears to be important for the maintenance of high diversity of tardigrades at the landscape scale. It provides a wider array of environmental conditions and resources (Guil and Sanchez-Moreno, 2013), as well as biotic interactions. This heterogeneity also promotes a wider diversity of bryophyte communities (Allen, 2002, de Brito Valente *et al.*, 2017) which in turn influences tardigrade composition (Richardson *et al.*, 2005).

This study shows that habitats separated by short distances of less than 100 m can have quite different tardigrade assemblages, since the environmental conditions are distinct. Furthermore, within the same habitat, different bryophyte hosts can vary considerably in tardigrade composition. When looking for biogeographical patterns at regional or continental scales, it is important to take account of this. Wherever possible, samples should be compared from the same kind of habitat, and the same bryophyte hosts sampled. Sampling from different habitats and hosts is likely to result in large differences being found which are potentially unrelated to coarse-scale biogeography.

Conclusions and recommendations

Quantitative comparisons of tardigrade community structure are currently too few to explain tardigrade distribution patterns. In part, such studies are hampered by the taxonomic difficulties of naming tardigrade species. It would help to advance ecological studies if taxonomists provided more accessible resources to improve the determination of named species as well as the description of new species. Alternatively, teams of ecologists and tardigrade taxonomists could work together more than currently happens to conduct research at the species level. In time, molecular techniques could facilitate ecological studies of tardigrades, but the risk is that they become divorced from taxonomy and rely entirely on bioinformatic comparisons.

However, OTUs worked well in this study, showing that effective ecological studies can be carried out without the naming of species. Nevertheless, it was still a time-consuming process to assign all the specimens to OTUs.

In this study, habitat influenced tardigrades more than bryophyte host, but both were important. More replicate sampling across a wide range of bryophyte hosts in habitats around the world are needed to refine our understanding of the interaction between host and habitat shown in this study.

Studies of tardigrade biogeographical patterns at regional or continental scales should compare similar habitats and, where possible, the same bryophyte hosts, since different habitats and hosts are likely to vary considerably within even the same landscape.

The structure of tardigrade communities at fine spatial scales in an Andean *Polylepis* forest

Introduction

One of the challenges facing contemporary ecology is understanding biodiversity patterns in very small organisms (Prosser *et al.*, 2007). Little is known about the distribution of these organisms over different spatial scales, or the mechanisms driving patterns in their distribution across different environments (Green *et al.*, 2004, Fierer and Jackson, 2006b). Whilst there are a number of apparently general, scale-related patterns in ecology, such as species-area and species-energy relationships (Rosenzweig, 1995, Lawton, 1999, Andrew *et al.*, 2003, Brehm *et al.*, 2003, Bonn *et al.*, 2004, Davies *et al.*, 2004, Dimitrakopoulos and Schmid, 2004, Gaston *et al.*, 2005, McAbendroth *et al.*, 2005, Rahbek, 2005), it is unclear how much such patterns apply to meiofauna – animals smaller than 2 mm (Fontaneto *et al.*, 2006). Since community composition of macroorganisms is easier to describe than that of microscopic organisms, the majority of studies have focused on studying species diversity of such macroorganisms (Green and Bohannan, 2006, Nemergut *et al.*, 2011, Feinstein and Blackwood, 2012).

Despite being poorly known in many cases, it is clear that meiofauna can comprise a significant fraction of the biodiversity in many ecosystems and play important roles in ecosystem function, as part of trophic webs, and in energy and nutrient transfer (Sohlenius *et al.*, 2004, GuilSanchez-Moreno *et al.*, 2009). However, despite their abundance and ubiquity, the roles of these organisms are often poorly defined. In fact, even the basic taxonomy of meiofauna and their spatial distribution patterns remain incompletely known. One of those overlooked groups is the phylum Tardigrada.

Tardigrades represent a convenient meiofaunal group for study. They are relatively abundant in a wide range of situations and are found from the equator to the poles, in both aquatic and terrestrial environments. They are potentially interesting ecologically as they share a common evolutionary history with other multicellular animals but have similar environmental needs and biological characteristics to many unicellular organisms (Guil and Giribet, 2012, Guil and Sanchez-Moreno, 2013). Their frequent ability to enter a dormancy stage provides them with the ability to survive desiccation, significant temperature variations and other extreme conditions (Jönsson *et al.*, 2005,

Horikawa *et al.*, 2006b, HorikawaKunieda *et al.*, 2008, Rebecchi *et al.*, 2009, Guil and Sanchez-Moreno, 2013). In addition, although tardigrade studies are limited practically by processing time (associated with sorting and mounting any microscopic organisms), their taxonomy is relatively well documented and updated checklists taxa and associated keys are regularly published (Guidetti and Bertolani, 2005).

Information about tardigrade distribution patterns comes mostly from information found in taxonomic descriptions, however, resulting in a lack of information about tardigrade diversity and abundance at all spatial scales. Some species are apparently observed in many different parts of the world (McInnes *et al.*, 2001), whilst others have only been reported from a single locality (Bertolani and Rebecchi, 1996, Ito, 1999, Guidetti and Bertolani, 2001). It is not clear whether this reflects genuine differences in distribution or merely results from insufficient material, although, in many cases, the latter appears likely. Very few studies have attempted to examine finer-scale distribution patterns in tardigrades (GuilSanchez-Moreno *et al.*, 2009), and how representative these are is unclear. One feature of tardigrades is that they are apparently very variable in abundance at fine spatial scales, which can result in patchy datasets with many samples containing few or no organisms. Meyer (2006) and Glime (2017), emphasised the importance of pilot studies to determine appropriate sampling strategies in such cases, but few studies have done this, or systematically explored the pattern or its practical consequences in nature (Meyer, 2003, Meyer, 2006).

In general, it has been suggested that the distribution of animals of microscopic size is highly influenced by the interaction between macroenvironmental characteristics (climate, soil, etc.) and micro environmental factors (vegetation, bryophytes and leaf litter). It has been widely proposed that tardigrade distribution is highly influenced by microhabitat conditions (GuilHortal *et al.*, 2009, GuilSanchez-Moreno *et al.*, 2009).

However, ecological studies at small scales are very limited (GuilSanchez-Moreno *et al.*, 2009) with most focusing on the impact of meso- and macro-scale factors (Dastych, 1988, Kathman and Cross, 1991). Although, little is known about tardigrade habitat associations (GuilSanchez-Moreno *et al.*, 2009) it is commonly assumed that species-specific habitat patterns do exist in these animals (Ito, 1991, Ito, 1993, Ito, 1995, Bertolani and Rebecchi, 1996, Ito, 1997, Guidetti *et al.*, 1999, Ito, 1999, Guidetti and Bertolani, 2001, GuilSanchez-Moreno *et al.*, 2009). However, many existing studies have

concentrated their efforts on altitudinal variations over relatively large spatial scales (Bartős, 1939, Guidetti *et al.*, 1999, Collins and Bateman, 2001) and very few have conducted quantitative sampling or statistical analyses to determine relationships between tardigrade species diversity, abundance and environmental factors (GuilHortal *et al.*, 2009). In addition, despite the fact that most studies of tardigrade diversity have focussed on the fauna on mosses and lichens (Glime, 2017), none of these studies have explored the extent to which tardigrade taxa are host specific. It is not known with certainty whether there is a specific epifaunal association with a particular kind of host, or if most taxa are relative generalists in this regard. Rarer tardigrades may, for example, be associated with specific hosts, but the extent to which this is the case remains unclear.

This study explores fine scale variation in tardigrade assemblages in an Andean *Polylepis* forest. I explore whether different bryophyte hosts differ consistently in the species of tardigrade they support, whether there is spatial structure to tardigrade assemblages within a microhabitat type and attempt to estimate the number of samples required to obtain a complete picture of tardigrade diversity at the site scale. This is the first such detailed exploration of Andean tardigrades, and indeed one of the first to investigate such factors in these organisms anywhere in the world.

Methods

The study was carried out in a forest consisting entirely of *Polylepis* trees located at 3,575 m in the buffer zone of El Ángel Ecological Reserve, Carchi Province in northern Ecuador (Fig. 5.1). *Polylepis* is the dominant tree in such habitats, which have long been recognised as a key vegetation type close to the Andean treeline (Fjeldså, 2002). These woodlands occur higher than any others, most commonly on mountain slopes, in deep canyons and ravines, and often in boulder fields or on steep rocky terrain (Kessler, 2002, Kessler *et al.*, 2014). The trees give shelter to several species of epiphytic vascular plants, mosses and lichens, as well as animals, including mammals and birds (Kessler, 2002). The study site experiences very little seasonality as it is close to the Equator, with humid conditions all year round. At the time of sampling (10–14h00), the average soil-temperatures ranged between 12–14 °C, but night-time temperatures are likely to fall below 5 °C (Balbina Ramsay, *personal observations*, 2011).

The site was relatively flat with organic soil, decaying wood and leaf litter; the forest floor was grazed by livestock and occasionally visited by tourists from a nearby hotel. Samples were collected in shaded areas, typical of this forest type.

I sampled tardigrades living in bryophytes on the ground. Additional bryophytes were present on the contorted trunks of the trees and on the branches and twigs of the canopy, but these were not sampled in order to minimise the effects of other variables (e.g. height, substrate, pH) on tardigrade communities. Within an area of 400 m², I collected five replicate samples of approximately 4 cm³ uncompressed volume from pure monospecific patches of five bryophyte species ("pure hosts"): *Leptodontium longicaule* Mitt., *Pleurozium schreberi* (Brid.) Mitt., *Thuidium delicatulum* (Hedw.) Schimp., *Zygodon nivalis* Hampe, and *Chiloscyphus latifolius* (Nees) J.J. Engel & R.M. Schust. The growth form and structure of each of these bryophytes is shown in Fig. 5.2 also collected 25 samples from an area of intimately mixed *Thuidium delicatulum* and *Pleurozium schreberi* ("mixed host") at 0.5 m intervals. No other species of bryophytes were growing on the ground in the sampled area. Samples were air-dried in individual paper envelopes, and stored at 10–25 °C until tardigrades were extracted.

In the laboratory, dried samples were rehydrated in tap water for 16–24 h. Rehydrated samples were shaken and passed through a 38 µm mesh sieve. Material retained by the sieve was searched for tardigrades using a Kyowa SDZ-PL stereoscopic microscope with 30–40x objectives (Kyowa, Japan). Tardigrades were mounted individually on microscope slides under cover slips in Hoyer's mounting medium. The identification of tardigrades was done to Operational Taxonomic Units (OTUs) with a Leica DMLB microscope with 40x and 100x objectives (the latter with immersion oil), using Guidetti and Bertolani (2005), Marley *et al.* (2011), and Degma (2013). Tardigrade taxa were also classified into four feeding groups according to Hallas and Yeates (1972), and personal observations of tardigrades by Balbina Ramsay and Nigel Marley (Fig. 5.3).

Overall tardigrade numbers, OTU richness and Shannon diversity of samples were analysed using one-way General Linear Model (GLM ANOVA) or Kruskal-Wallis Tests, dependent on the outcome of a Shapiro-Wilks Test for normality. These statistical tests were carried out with R version 3.3.3 (R Core Team, 2017).

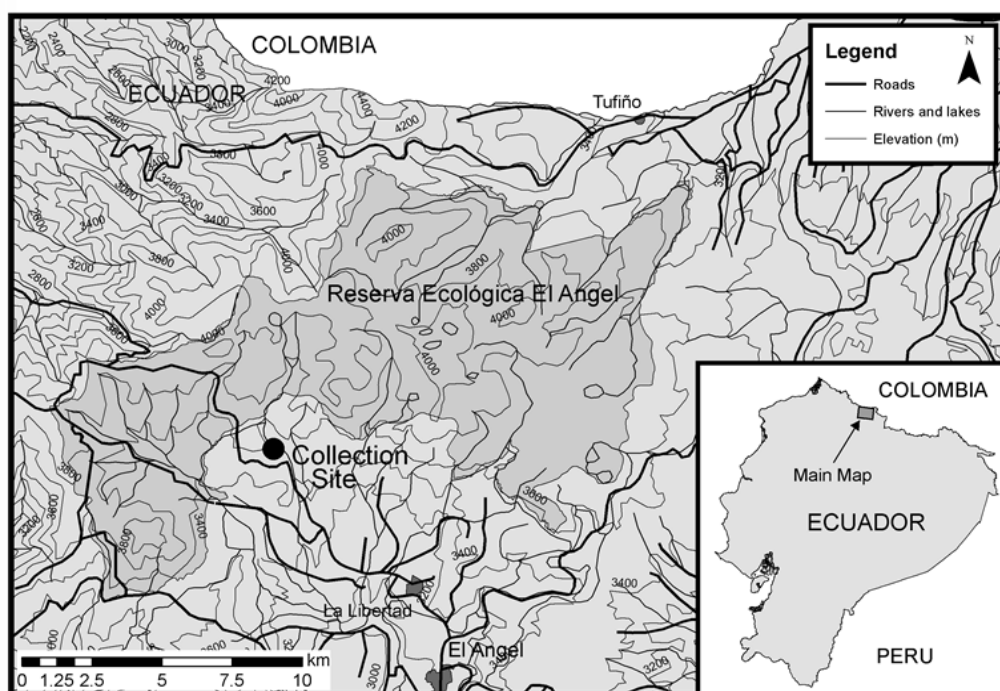
Species accumulation curves for tardigrade OTUs richness (*S*) for pure host and mixed host samples estimated the number of samples needed to fully characterize tardigrade

communities. I used Estimate S (Version 9, R.K. Colwell, <http://purl.oclc.org/estimates>) to plot the cumulative number of OTUs found as a function of sampling effort (species accumulation or rarefaction curves). For sample-based data, the estimator of asymptotic richness was Chao 2 (Chao, 1984, Chao, 1987). The species accumulation curve was extrapolated to 50 samples (double the number of samples taken in each case, and the maximum extrapolation advised in the software user manual).

The OTU composition of samples was compared using non-metric Multidimensional Scaling (MDS) in Primer 6 (Primer-e, Plymouth, UK), on square-root transformed OTU count data. The graphical output of this approach positions samples with similar composition close together and samples with more different composition further apart. Statistical differences in sample composition were determined by permutational ANOVA (PERMANOVA) using the PERMANOVA+ add-on to Primer 6. PERMANOVA is sensitive to differences in the dispersion of data (Anderson et al., 2008) and so an additional test, when significant differences were identified by PERMANOVA, was carried out to identify any significant differences in dispersion between groups, using PRIMER's PERMDISP.

To determine whether OTU composition (measured as percentage similarity in tardigrade OTU composition of pairs of samples) could be predicted by physical distance between the samples, reduced major axis (RMA or Model II) regression was conducted in R using the package "lmodel2" on the mixed host samples, using a one tailed test (Legendre, 2018).

Figure 5.1. Location of the collection site from El Ángel Carchi Ecological Reserve, Carchi Province in northern Ecuador at 3575 m elevation.



Results

Across all fifty samples (mixed and pure hosts combined), I identified 51 tardigrade OTUs (Fig. 5.3). Some tardigrades found in this study represent new taxa (e.g. *Adropion cf grevenie*, *A. cf tricuspidatum*, *Hypsibius* sp nov 200, *Hypsibius* sp nov 201, *Isohypsibius saulrodgersi* sp nov and *Isophypsibius* sp 1 210). *Macrobiotus* 210 is the only taxon present in all bryophyte species examined (pure and mixed). Some rare OTUs observed in this study occurred as single individuals, such as *Adropion cf grevenie* and, *A. cf tricuspidatum*.

Forty-three tardigrade OTUs, comprising 692 specimens, were found across the pure host samples (Fig. 5.3). Individual samples contained 1–74 individuals and up to 16 OTUs. Eutardigrades outnumbered heterotardigrades in abundance (660 vs. 32 individuals) and taxon richness (32 vs. 1 taxa). Thirty-three OTUs were found across 648 specimens in the mixed host samples. Individual samples here contained 5–62 individuals and up to 17 OTUs. Eutardigrades again outnumbered heterotardigrades in abundance (623 vs. 25 individuals) and taxon richness (30 vs. 2 taxa). Across all the samples, the tardigrade taxa were classified into 25 microbivores, 13 omnivores, 12 herbivores and one strict carnivore.

Tardigrade abundance was higher in pure host samples than in mixed host samples (Table 5.1). Mixed host samples had the highest OTU richnesses. Pure host samples of *Pleurozium schreberi* had the highest abundances and diversity indices whilst *Chiloscyphus* had the lowest in all three cases (respectively: Shapiro Wilks $p \leq 0.001$, Kruskal Wallis $df= 5$, $X^2= 28.315$, $p < 0.001$; Shapiro Wilks $p= 0.011$, Kruskal Wallis $df= 5$, $X^2= 25.428$, $p < 0.001$; Shapiro Wilks $p= 0.848$, ANOVA $F_{5,44}= 15.743$, $p < 0.001$; Table 5.1). The other hosts had intermediate levels of these descriptors.

The sample-based rarefaction curves for 25 mixed host and 25 pure host samples did not reach asymptotes of OTU accumulation, not even when extrapolated to 50 samples in each case (Fig. 5.4). The complete overlap of 95% confidence intervals for the rarefaction curves indicate that no significant differences in OTU accumulation exist between the mixed host and pure host samples.

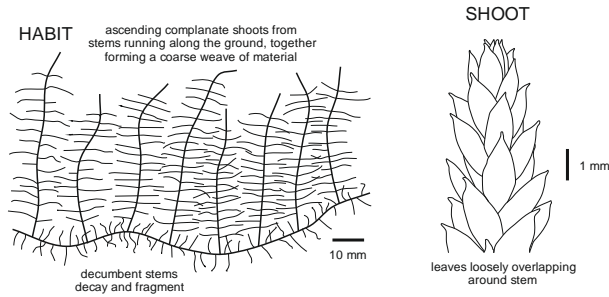
All host pairings had significantly different tardigrade compositions (PERMANOVA, $p = 0.001$ to 0.049), except between *Leptodontium* and *Zygodon* ($p=0.123$; Fig. 5.5 and Table 5.2). The dispersion of *Zygodon* samples in the analysis was much greater than that of the other samples (PERMDISP $p = 0.011$); and the other samples were not significantly different.

The similarity in distributions of OTUs across samples is depicted in Fig. 5.6. The cluster of OTUs in the centre of the figure, such as *Adropion* 300, *Diphascon* 311 and *Macrobiotus* 210, represent the most abundant OTUs, which were found across most host types, grouped together and listed at the top of Fig. 5.3. OTUs located around the periphery of the figure were less abundant and restricted to fewer hosts and samples.

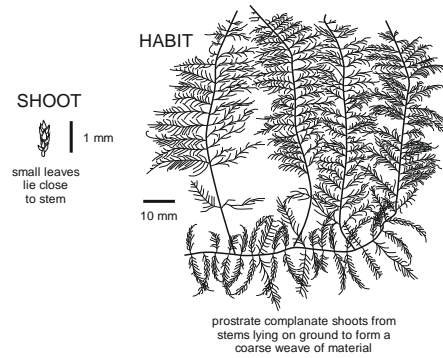
There was no significant relationship between physical distance and tardigrade composition in the mixed host samples (RMA regression $R^2= 0.006$; $p= 0.098$; Fig. 5.7).

Figure 5.2. The habit and detailed morphology of the five bryophytes collected in this study: *Leptodontium longicaule*, *Pleurozium schreberi*, *Thuidium delicatulum*, *Zygodon nivalis* and *Chiloscyphus latifolius*.

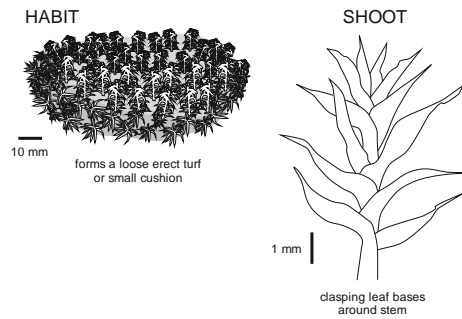
Pleurozium schreberi



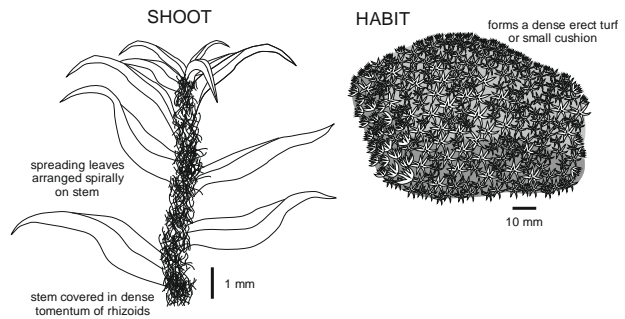
Thuidium delicatulum



Leptodontium longicaule



Zygodon nivalis



Chiloscyphus latifolius

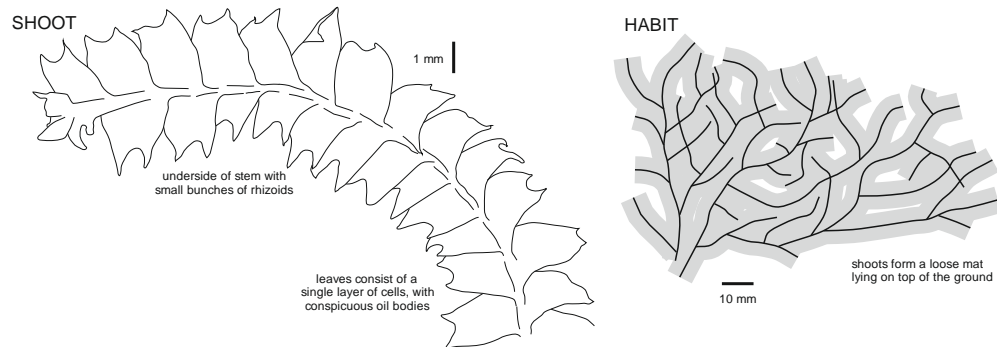


Table 5.1. Descriptors of tardigrade communities in host samples: N = total number of tardigrades, Overall S = total number of OTUs in all samples, S = mean \pm sd number of OTUs, and H' = mean \pm sd Shannon Index based on OTUs. Means sharing a letter within a column were not significantly different.

Host	Sample n	N	Overall S	S	H'
<i>Pleurozium</i> + <i>Thuidium</i>	25	25.9 ^{bc} \pm 15.9	33	8.9 ^b \pm 3.1	1.9 ^{ab} \pm 0.3
<i>Thuidium</i>	5	30.6 ^b \pm 5.2	18	9.0 ^b \pm 2.9	1.8 ^{ab} \pm 0.4
<i>Pleurozium</i>	5	56.0 ^a \pm 10.7	32	15.4 ^a \pm 1.7	2.2 ^a \pm 0.2
<i>Leptodontium</i>	5	39.2 ^b \pm 22.2	22	10.4 ^b \pm 4.3	1.9 ^{ab} \pm 0.3
<i>Zygodon</i>	5	9.6 ^{cd} \pm 3.0	20	6.0 ^b \pm 2.0	1.6 ^b \pm 0.3
<i>Chiloscyphus</i>	5	3.0 ^d \pm 1.9	5	2.0 ^c \pm 0.7	0.6 ^c \pm 0.4
Grand Total	50	26.8 \pm 19.2	51	8.7 \pm 4.2	1.7 \pm 0.5

Table 5.2. Similarity in tardigrade OTU composition within and between sample types. Diagonals in red represent percentage similarity within host samples. The remaining figures (in black text) represent percentage similarity between host samples. Significance of pairwise PERMANOVA analyses is shown by shading (NS none, $p < 0.05$ light grey, $p < 0.01$ dark grey, $p \leq 0.001$ black).

Host	<i>Pleurozium</i> + <i>Thuidium</i>	<i>Thuidium</i>	<i>Pleurozium</i>	<i>Leptodontium</i>	<i>Zygodon</i>	<i>Chiloscyphus</i>
<i>Pleurozium</i> + <i>Thuidium</i>	49.626					
<i>Thuidium</i>	35.715	46.427				
<i>Pleurozium</i>	37.543	46.547	59.471			
<i>Leptodontium</i>	32.571	41.161	48.302	49.553		
<i>Zygodon</i>	24.231	24.004	25.889	27.068	17.922	
<i>Chiloscyphus</i>	5.7476	12.407	6.2785	6.3845	5.7882	50.503

Figure 5.3. Tardigrade OTUs in 50 samples of bryophytes from a *Polylepis* woodland at 3575 m in Carchi Province, Ecuador. The area of the circles represents the number of samples occupied (left panel) or the mean abundance within the relevant samples (right panel), with a legend at the foot of each panel. coloured circles represent a different tardigrade feeding habits, yellow for omnivore, blue for microbivore, green for herbivore and red for carnivore. OTUs named “cf” and “sp.” followed by a number refer to recognizable morphospecies, some of which are new to science, and are to be described in the future. The “combined pure hosts” columns represents the tardigrades from all the pure host samples added together.

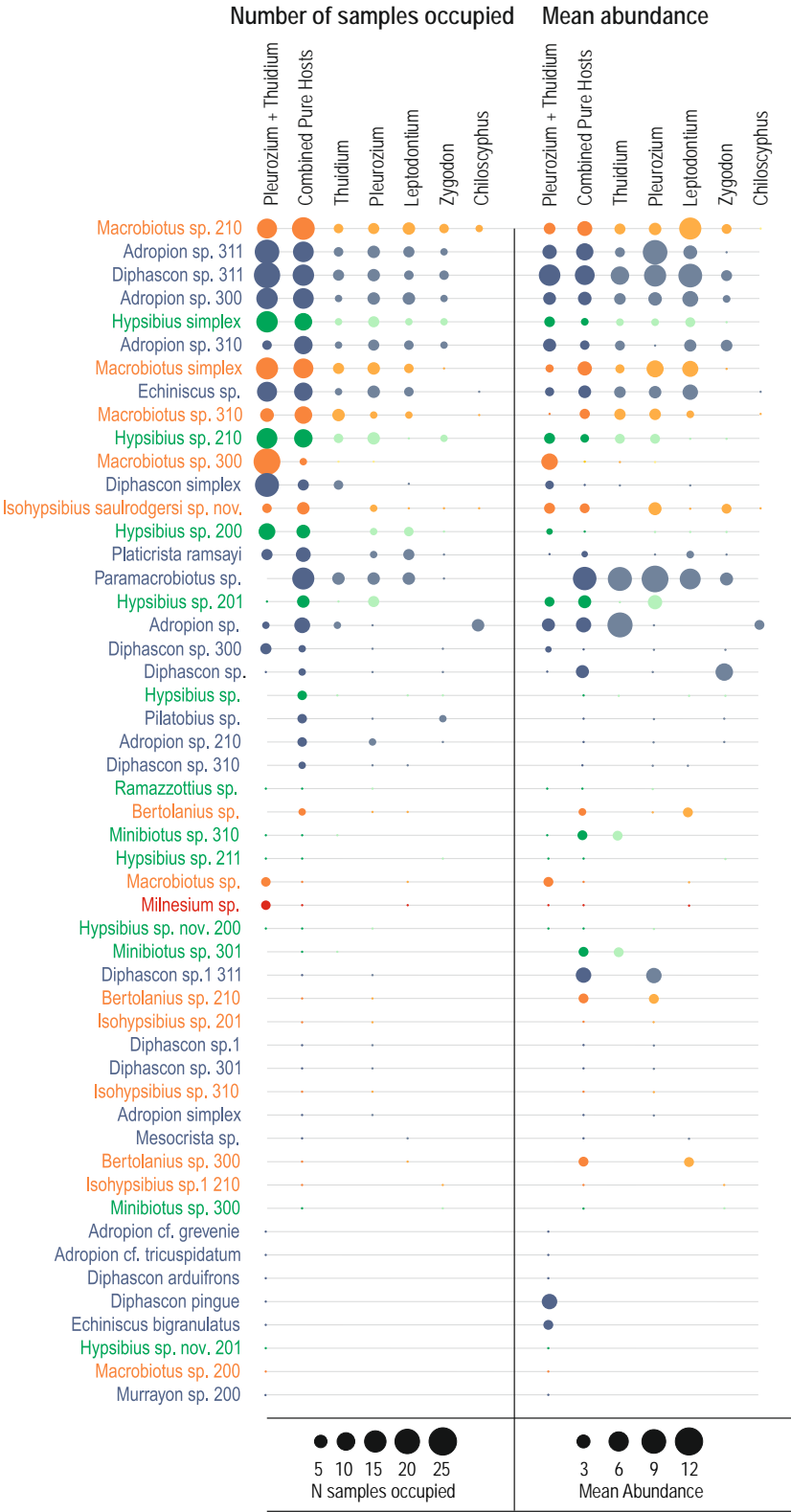


Figure 5.4. Species accumulation curves for tardigrades species richness (S) on the floor of a *Polylepis* woodland in the north of Ecuador: (A) a mixed substrate of *Pleurozium* and *Thuidium* ($n=25$); and (B) five samples each from pure substrates of five different bryophyte species (total $n=25$). The continuous line represents the sample-based rarefaction curve for the data set (25 samples), while the dashed line represents the predicted rarefaction curve for up to 50 samples. The shaded areas are bounded by the upper and lower 95% confidence limits for the estimates. (C) Estimates of the species richness asymptote for mixed *Pleurozium* and *Thuidium* samples (orange) and pure bryophyte hosts (blue), using the Chao2 estimator.

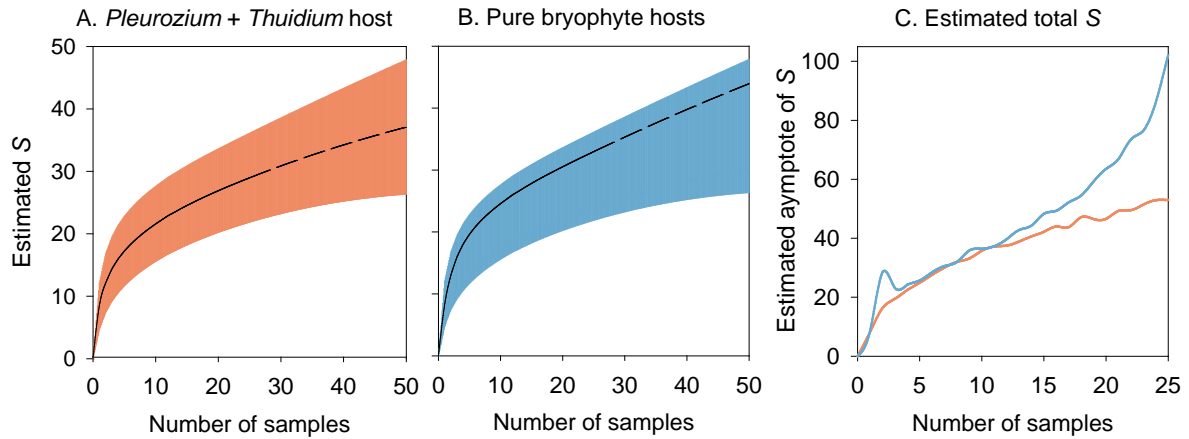


Figure 5.5. MDS ordination of host samples, based on tardigrade OTU composition, for mixed (*Pleurozium* + *Thuidium*) and pure hosts. Samples located close together in the figure had similar compositions of tardigrades, whereas those further apart were more different in composition.

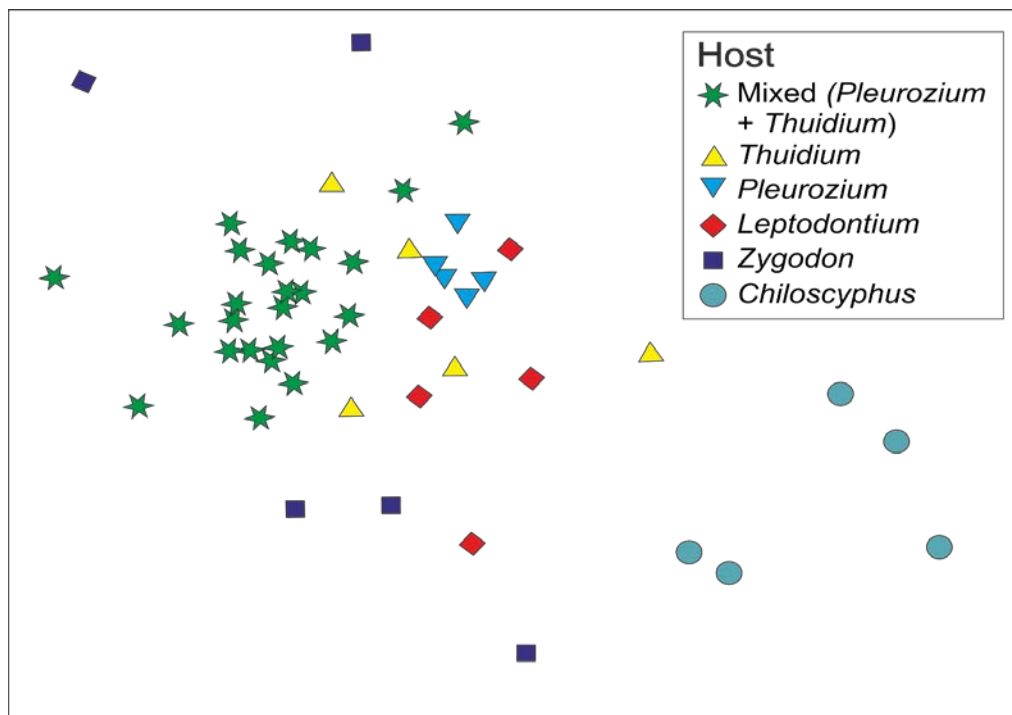


Figure 5.6. MDS ordination of OTUs within the samples. OTUs located close together in the figure tended to be found in the same samples, whereas OTUs far apart in the ordination were found mostly in different samples. The OTUs in the central part of the figure were the most abundant and found in a wider range of samples.

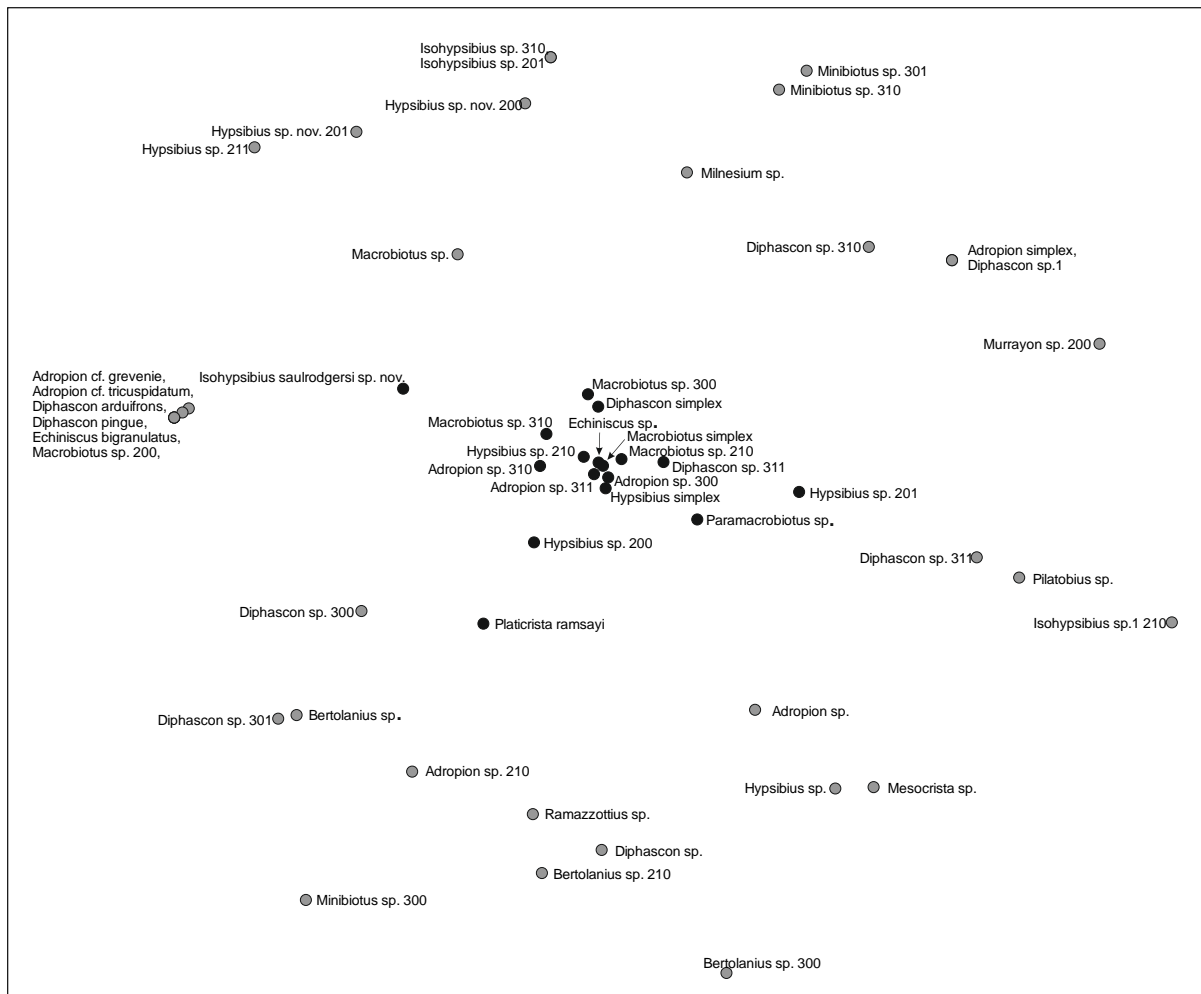
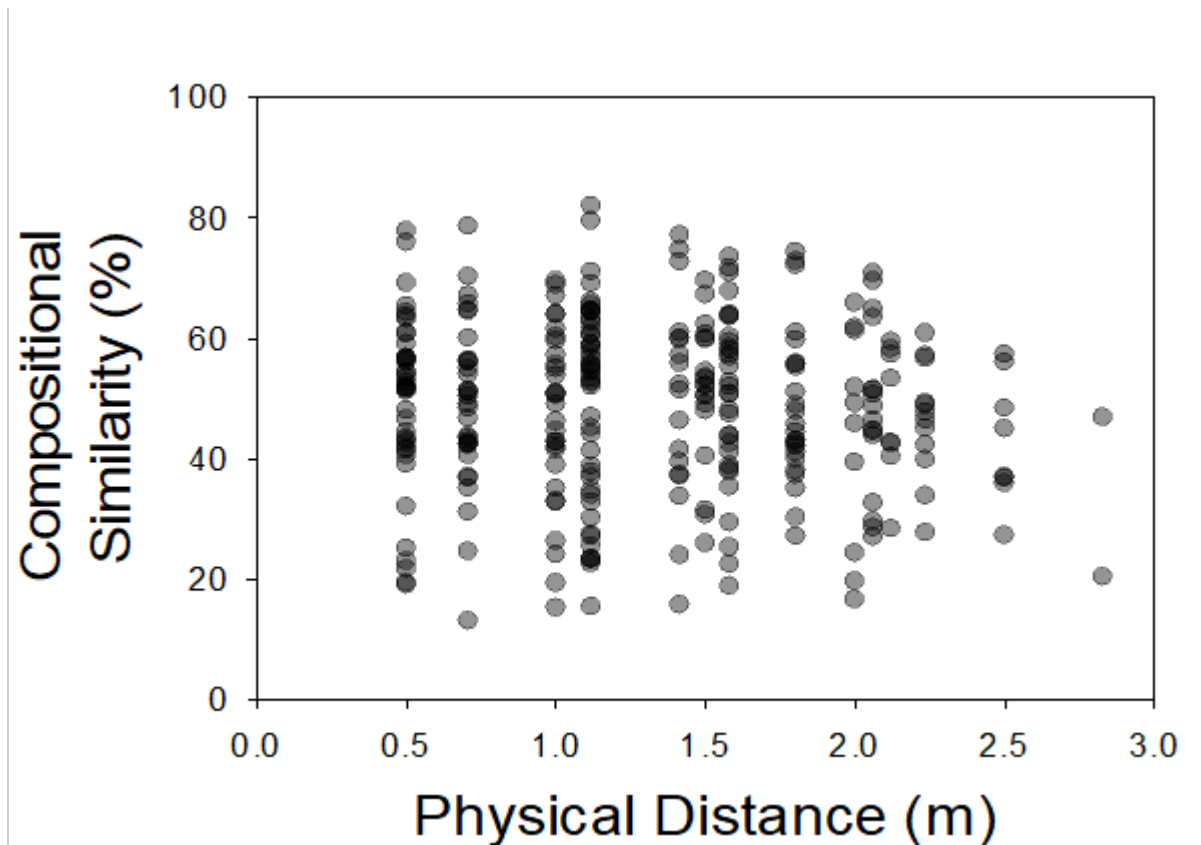


Figure 5.7. Relationship between physical distance between pairs of mixed host (*Pleurozium* + *Thuidium*) samples and their similarity of tardigrade composition.



Discussion

Tardigrade abundance and species richness varied considerably between the samples, a pattern that has been shown in the relatively few other studies that have sampled tardigrades quantitatively (Meyer, 2006, GuilSanchez-Moreno *et al.*, 2009, KaczmarekGołdyn *et al.*, 2011). In general, bryophyte samples of tardigrades are known to vary in the number of individuals and species richness (Maucci, 1980, Kathman and Cross, 1991, Degma *et al.*, 2017). However, it is difficult to compare tardigrade diversity across different studies where sampling has not been standardised, or even properly described. It would be useful for studies collecting quantitative data on tardigrade composition to describe their methods in detail. Furthermore, despite the practical difficulties in standardising samples of complex, three-dimensional host organisms, I propose that sampling should aim to collect consistent volumes of uncompressed host material. Based on my study, I propose a standardised sample for bryophytes (mosses, hepatics and liverworts) and lichens of the equivalent of a sphere approximately 4 cm diameter, which represents approximately 4 cm³ in volume.

Terrestrial tardigrades fall into two Classes: Eutardigrada and Heterotardigrada. In our samples, eutardigrades were high in OTU richness while heterotardigrades presented low richness. This matches patterns found in quantitative studies of tardigrades in central Spain (GuilSanchez-Moreno *et al.*, 2009, Guil and Sanchez-Moreno, 2013). Eutardigrade diversity is often highest in humid environments, while heterotardigrades are most diverse in drier conditions (Bertolani *et al.*, 1987, Ito, 1993, Ito, 1999, GuilSanchez-Moreno *et al.*, 2009). In some previous quantitative studies of tardigrades, heterotardigrades have been found to be more abundant than eutardigrades e.g., (GuilHortal *et al.*, 2009, GuilSanchez-Moreno *et al.*, 2009, Guil and Sanchez-Moreno, 2013), though the relative abundances of these Classes vary considerably (Maucci, 1980, Kathman and Cross, 1991, Degma *et al.*, 2017, Glime, 2017). In contrast, my samples from *Polylepis* forest had more individuals belonging to the Eutardigrada than the Heterotardigrada. *Polylepis* forests in Ecuador are very humid environments (Richardson *et al.*, 2005), where a higher overall abundance of individuals of Eutardigrada might be favoured, given the higher taxon richness of this Class in humid habitats more generally.

Macrobiotus species were abundant in most samples, and this genus is the most common resident of bryophytes worldwide (McInnes *et al.*, 2001, Schuster and Greven, 2007, Glime, 2017). Other tardigrades with a global distribution were also common in our samples, such as *Diphascon*, *Hypsibius* and *Paramacrobiotus* (Pilato and Sperlinga, 1975). Interestingly, several OTUs of *Bertolanius* were present in the samples. This genus has been considered a Holarctic genus (Hansen *et al.*, 2017), but this study extends the presence of the genus into the equatorial mountains of South America. Apart from the biogeographical patterns of genera, it is difficult to compare the tardigrade composition of *Polylepis* forest in more detail because there are so few studies of tardigrade assemblages.

Some tardigrade taxa in our forest samples were sparse, in that they occurred at in very low numbers (e.g., *Adropion* cf. *grevenie*, *Adropion* cf. *tricuspidatum*, *Diphascon arduifrons*, *Echiniscus bigranulatus*). Many other reports of tardigrade sampling have found sparse taxa (Ramazzotti and Maucci, 1983). In general, there are several different forms of sparsity (Rabinowitz, 1981), and therefore several different potential explanations for the low abundance and occupancy of taxa in our samples. The potential

explanations include fluctuating resources limiting tardigrade numbers, poor resources offered by the host, and the rarity of specific microenvironmental conditions and habitats (Rabinowitz, 1981). Tardigrade numbers can also be reduced by disease, predation (sometimes by other tardigrades, such as *Macrobiotus* which feeds on other tardigrades: (Kinchin, 1994), and interactions with other meiofauna, including other tardigrades (Sohlenius and Bostrom, 2006). Furthermore, although cryptobiosis helps tardigrades to survive adverse conditions, it is energetically costly and is known to limit reproduction (McInnes, 1994, Suzuki, 2003).

Some taxa were clearly associated more with some hosts than others. The physical structure and chemical composition of particular hosts might determine the abundance of tardigrades. Tardigrades were more abundant and diverse in mosses from the *Polylepis* woodland floor than in the liverwort. Mosses are more structurally complex than liverworts, growing vertically or horizontally, and forming mats or cushions (Gradstein *et al.*, 2001). Thus, the more complex three-dimensional structures of the mosses in our study might provide conditions for a wider number, and potentially a greater diversity, of tardigrades than the simpler structures of the liverwort, *Chiloscyphus*—in a similar way to that suggested for terrestrial and freshwater invertebrates. Suzuki (2003) also found that some tardigrades were favoured by the intricate structure of mosses.

In my study, *Pleurozium* had the highest , and whilst the structurally simple *Chiloscyphus* had the lowest, other hosts were intermediate (including the combined samples of *Pleurozium* and *Thuidium*). *Zygodon* had the lowest tardigrade abundance and diversity of the mosses in this study, but the samples varied in the tardigrade taxa that were present (though drawn from a similar pool to that of *Pleurozium* and *Thuidium*). Mosses provide different habitats for tardigrades (see Fig. 2). Although *Pleurozium* and *Thuidium* have a similar pleurocarpus form, *Thuidium* has much smaller leaves arranged tightly around the stem. *Zygodon* and *Leptodontium* appear structurally similar at a coarse scale, but *Zygodon* has dense fine hairs (rhizoids) covering the stem. It is not clear to what extent the structural characteristics of hosts affect the abundance and diversity of tardigrades within them, but our results suggest that further exploration of this aspect would be worthwhile.

Certain bryophytes deter herbivores with phenolic compounds (Swain and Hillis, 1959), and liverworts often defend themselves with terpenoids and lipophilic compounds located in oil bodies (Markham, 1988, Zinsmeister and Mues, 1988, Asakawa, 1999, von Schwartzenberg *et al.*, 2004). *Chiloscyphus*, along with other liverworts, has oil bodies within the leaves and gives off a characteristic odour, which may represent a form of chemical defence against herbivory (Asakawa, 1999, Glime, 2006). As discussed above, *Chiloscyphus* had the lowest tardigrade abundance and diversity in our study, with only widely-distributed tardigrade taxa and no evidence of specialist species. Amongst the mosses sampled in this study, *Pleurozium schreberi* has a reportedly higher content of phenolic compounds than *Thuidium delicatulum* (Glime, 2006). In an experiment with pill bugs (Armadillidiidae, Oniscidea), *Pleurozium schreberi* was the least preferred of the mosses on trial due to its high phenolic content (Smith *et al.*, 2001). In contrast, I found *Pleurozium* had the highest tardigrade abundance and diversity, across a wide range of taxa. This suggests that phenolic content is not just the only factor influencing tardigrade occupancy.

Only a few studies have looked for an association between tardigrades and their hosts but the results have been mixed. Bertolani's (1983) study found that hosts were not important, whilst other studies have suggested that particular tardigrades were linked to specific hosts (Degma, 2003, Degma and Pecalkova, 2003, Degma, 2006). Drawing conclusions from these studies is difficult because of the great variability in occupancy from sample to sample: often it is not clear from low sampling effort whether these animals show real preferences between hosts or just stochastic differences in occupancy. In addition, at present so little is known of tardigrade ecology and life history that explaining any apparent preferences would be rather speculative.

I found more microbivore OTUs than any other feeding group, with omnivore and herbivores being found in almost equal numbers. Only one strictly carnivorous tardigrade taxon was present in our samples, but did not impact on the number of herbivores in our samples. However, the presence of only one strict carnivore but thirteen omnivores suggests that the ability to utilise a varied diet, including plants, might be favoured in the *Polylepis* forest. Guil and Sanchez-Moreno (2013) is the only other study to date to consider trophic groups in natural tardigrade assemblages, but was limited by a relatively small number of samples and categorized tardigrades into

three feeding groups on the basis of the buccal apparatus and assumed feeding habits (Hallas and Yeates, 1972, Guidetti *et al.*, 2012). In most of these samples from central Spain, carnivores were the most species rich trophic group (although this included omnivores in their classification), followed by herbivores, whilst microbivores were the least species rich. Our results contrast markedly with this study. Although it is not clear why these differences exist and clearly more studies are required before patterns emerge and potential explanations can be developed.

Tardigrades were only sampled from the forest floor ignoring epifauna on trees. Tardigrades are known to inhabit a range of microsites within the forest including bryophytes and lichens on trunks, branches and twigs, as well as bark itself (McInnes, 1994). Therefore, the tardigrades found in this study may not represent the entire forest community in the forest. It is unclear to what extent tardigrade taxa are restricted to particular positions within the forest structure. Consequently, more studies are required to get a better understanding of tardigrade distribution in forest ecosystems. Nevertheless, many tardigrade taxa found in our samples from the forest floor have also been found in other studies of tardigrades on trees (GuilHortal *et al.*, 2009, GuilSanchez-Moreno *et al.*, 2009, Guil and Sanchez-Moreno, 2013)

An important finding of this study was the very high sample effort that was required to estimate tardigrade OTU richness: more than 50 samples would apparently be needed to do this with confidence. However, whilst such sampling effort in the field represents a few hours work, the subsequent laboratory work is very time consuming for such a number of samples (approximately from three to six months of processing, plus another month of identification), something which applies equally to ecological and taxonomic studies, if the aim is to characterise the tardigrade fauna. Clearly, whilst resource demands are high, without taking enough samples it is likely to be impossible to obtain an accurate picture of tardigrade assemblages. Common, widespread taxa are the most likely to be found, whilst rarer, potentially more interesting species may be overlooked. Comparing sites and studies only makes sense if the threshold for effective sampling is met. It is not clear whether the threshold of 50 samples suggested by our study is typical of that required to sample tardigrades in other habitats. This is such a fundamental issue that similar studies in other habitats are urgently required, as part of a wider effort to find effective ways to estimate tardigrade diversity at different scales

that is accurate, practical and feasible (Meyer, 2006). Furthermore, for taxonomic studies, greater sampling effort would be more likely to provide the number of individuals needed for the description of new species. Based on a detailed study of several tardigrade species, Stec *et al.* (2016) found that 6–40 individuals of each species were required to adequately describe morphological variation. Several species in our study did not reach these numbers, even with 50 samples.

In recent years, much effort has been dedicated to analysing patterns of biodiversity for microscopic organisms through the analysis of distance-decay relationships, taxon-area relationships, and local: global taxon richness ratios. Despite this attention, patterns of micro-organism diversity at continental and global scales are still unclear (Green *et al.*, 2004). Studies at finer scales can complement those broader studies (Green and Bohannan, 2006). In our samples from widely distributed bryophytes, OTU assemblages were not driven by physical distance over small scales, and did not show spatially predictable patterns at this scale. Thus, it seems that fine-scale differences in environmental conditions (including the distribution of host bryophytes) is much more important in determining tardigrade composition than distance. In other words, the composition of tardigrades in a forest can vary as much between neighbouring bryophytes as between more distant ones.

Conclusions

This work adds to a small number of quantitative studies of tardigrade assemblages. The sparsity of some taxa and the variability in numbers from sample to sample, suggest that caution is required in interpreting results from studies which rely on a handful of samples from a locality. Using samples standardized to approximately 4 cm³, our study clearly showed that at least 50 samples are required to estimate tardigrade diversity effectively in *Polylepis* forest. I therefore propose that future quantitative studies should standardize the sampling efforts using replicate samples of approximately 4 cm³ (and report in detail the precise sampling strategy). More studies are required to show whether the requirement of at least 50 samples is typical of other habitats. Some tardigrades were restricted to certain hosts, and so collecting from a range of different hosts is recommended in order to obtain a representative picture of tardigrade diversity.

The widely-dispersed, inaccessible taxonomic literature on tardigrades impedes the naming of tardigrades at species level or genus level (Krell, 2004): information is scattered through time across many journals, some difficult to obtain outside of the host country or the world's largest libraries, and written in a range of languages. Some publications, especially older ones, do not include crucial diagnostic information, and even though it is sometimes possible to deduce features not formally described in the text from drawings and photos, other times access to the type specimen is needed to confirm the characteristic feature. Occasionally, even type specimens do not demonstrate some of the diagnostic characteristics needed for unequivocal identification, for example, because of inconvenient positioning on the mounted slide. Furthermore, the Linnean Shortfall in tardigrades—that many tardigrade species remain uncollected, unprocessed and/or undescribed—means that some specimens in a sample (e.g., from *Polylepis* woodland in the Andes) might belong to species new to science. A thorough review of the literature might reveal this, but an additional step is needed to publish a valid description of the new species. This publication process can be time-consuming, particularly since some journals have policies to avoid papers presenting just one new species at time: e.g., *Zootaxa* discourages manuscripts dealing with a single species description (though there is editorial discretion for species of particular significance).

In ecological studies like those reported in this thesis, all specimens must be identified for the data to make sense, and unidentified specimens cannot be ignored. By definition, comparisons of abundance and diversity must count all individuals and attribute them all to suitable taxonomic units—ideally, species. For the reasons previously discussed this is practically impossible for most ecological studies of tardigrades. Partly this is the reason why quantitative studies comparing tardigrade community structure are scarce. In addition, with so many individual specimens involved, each separately mounted on a microscope slide, it is much more likely that some of them will be positioned on the slides in ways that obscures some of their diagnostic features.

The character matrix approach, illustrated by the chapter on *Isohypsibius*, helps to solve several of these problems. It brings together the diverse literature into one place, with one researcher or team of researchers working on behalf of the rest of the scientific community to obtain, translate, interrogate and summarise the diagnostic features of all published descriptions (and where necessary, type specimens) for the taxonomic group in question. It does not rely on the availability of particular features for determination, as often happens in traditional keys which are often useless for specimens for which certain features are not visible, even when many other features are available.

Of course, since the *Isohypsibius* chapter currently represents the only genus-wide character matrix in the Tardigrada, ecological studies must advance largely without such aids for the time being. Fortunately, character matrices can be developed within the framework of individual studies, to ensure that taxonomic clarity, based on morphology, is maintained: that the same taxon is readily identified whenever it is found in a set of samples, and not confused with other taxa. Other ecological studies have used OTUs as an effective and quicker way of comparing samples than species level (Hackman *et al.*, 2017), such OTUs proved valuable in the studies presented in Chapters 3–5 and revealed interesting ecological patterns and conclusions. Direct comparisons of OTUs from studies carried out by different authors, however, would not be possible. This is the principal disadvantage of the approach, and the reason why named taxa should still be the ultimate goal.

In this study, the use of certain diagnostic characteristics of the group, e.g., the number of macro- and micro-placoids was used to create a placoid formula which was diagnostic in itself for most OTUs. However, the use of taxonomic units should be treated cautiously to avoid overestimation of species richness (Krell, 2004). In places like the Andes, many tardigrades have yet to be described, and OTUs offer a way forward while species await formal naming and description. Several new species were found in this relatively limited study. In summary, then, the use of OTUs worked relatively well, and permitted the classification of taxa from large numbers of samples within a reasonable time frame.

In the future, molecular OTUs will be more commonly used when working with tardigrades, but developments are still in their early stages, especially when considering community-wide studies.

The variable nature of tardigrade populations in host samples was reflected in the proportion of empty samples in the quantitative studies described in this thesis. It was not possible to detect samples that did not contain any tardigrades during the fieldwork phase. High proportions of empty samples can have negative consequences on the statistical reliability of the analysis and the confidence of the conclusions reached. This was the case in the work presented in Chapter 3, where 59% of samples contained no tardigrades at all. Increasing the size of samples in the studies described in Chapters 4 and 5 reduced the numbers of empty samples substantially but did not eliminate them completely (4% in the landscape-scale study and none in the fine-scale study).

In this study, eutardigrades were much more abundant than heterotardigrades in the samples from *Polylepis* forest, as well as from páramo grassland and bog. This generally fits with the suggested preferences of eutardigrades for more humid environments (Hofmann, 1987, Ito, 1993, Ito, 1999). However, these are generalities, illustrated by the exclusive preference of a heterotardigrade, *Echiniscus* sp., for the wet conditions of a bog. More nuanced conclusions will be possible once more studies have been carried out in a range of habitats around the world. Comparisons made at the class level are interesting and might be informative when relating samples from different ecosystems, but the ecological resolution at this taxonomic level is low for comparisons made among sites belonging to the same ecosystem. In such cases, where the underlying environmental conditions are likely to be similar, the use of species or OTUs is the appropriate taxonomic level to bring out relevant similarities and differences.

Most common and abundant genus was *Macrobiotus*, this has been reported as the most common resident of bryophytes worldwide (Schuster and Greven, 2007, Glime, 2017). Other tardigrades with global distribution were also common in these samples, such as *Diphascon*, *Hypsibius*, *Paramacrobiotus* (Pilato and Sperlinga, 1975).

Polylepis forest provides a useful model habitat for studying tardigrade distribution patterns across a range of scales. It offers habitat with suitable conditions for many host organisms and tardigrade taxa. Compared with mixed forests, particularly in the Tropics, their monospecific tree composition simplifies the interpretation of fine-scale tardigrade distributions (Fjeldså and Kessler, 1996). Inside the forests, the trees provide a complex structure within which fine-scale distribution patterns can be studied. At the landscape scale, *Polylepis* forms woodland patches in a mosaic, mostly

with grassland, allowing good quality comparisons at this scale. But it is their habitat consistency across approximately 5500 m distance along the Andes that offers the most value to biogeographical studies. Few, if any, other habitats spans such a wide latitudinal range (Kessler and Schmidt-Lebuhn, 2006).

The thesis presents the first description of tardigrades from a *Polylepis* forest, and demonstrated microhabitat preferences of tardigrades, despite a difficult dataset because of high variability in occupancy and abundance. The three-dimensional structure of the forest influenced tardigrade composition. Although tardigrade abundance and taxon richness were not significantly different, samples from tree branches differed from those from the forest floor. The wide range of humidity within the forest structure, from the more humid, shaded forest floor to the drier, sunnier canopy, might promote a greater overall diversity of tardigrades within the forest. A new species was even discovered in a sample of papery bark from the *Polylepis* tree, representing a potential new type of microenvironment for tardigrades.

The more complex structure of a forest (compared with, for example, the surrounding grassland) provides a niche-rich environment, promoting overall tardigrade diversity. This has been shown with vertebrates and invertebrates in other forest ecosystems (Castaño-Villa *et al.*, 2014, Zellweger *et al.*, 2017).

In the initial study, tardigrade abundance, taxon richness and composition did not show statistical differences between bryophytes and lichens, but comparisons were hindered by the high variability in the dataset, especially the high proportion of empty samples (59%). (KaczmarekGoldynProkop *et al.*, 2011) had similar problems with interpretation in their European comparison of tardigrades in bryophytes and lichens. In the pilot study, described in Chapter 3, there were similar difficulties in comparing the tardigrade samples from soil, rock and trees: no significant differences were found but the noisy dataset might have obscured underlying differences.

The forest floor was the only structural component of the forest that could be directly compared with neighbouring habitats. *Polylepis* maintains its own environmental conditions, decoupling it to some extent from the environmental conditions elsewhere in the landscape. Forest humidity is higher, while temperatures are cooler and less extreme than in more open habitats nearby (Körner, 2012). These conditions promote

the abundance of suitable hosts, and *Polylepis* forest floors are characterised by carpets of bryophytes and lichens (Fjeldså and Kessler, 1996).

Bryophytes on a *Polylepis* forest floor had greater tardigrade abundance and diversity than bryophytes on the ground in grassland and bog habitats nearby (Chapter 4). The forest therefore provides a clear additional tardigrade biodiversity contribution at a landscape scale. This effect of *Polylepis* forests has already been recognised for plants and other groups of animals (Gareca *et al.*, 2010, Tinoco *et al.*, 2013, Bellis *et al.*, 2015). A similar effect is also likely to be true for other micrometazoan groups. Elsewhere, habitat heterogeneity has been shown to play an important role maintaining high diversity of tardigrades at the landscape scale (Guil and Sanchez-Moreno, 2013). Such heterogeneity offers a wide range of environmental conditions and resources, promoting a wider diversity of bryophyte communities (Allen, 2002, de Brito Valente *et al.*, 2017) which in turn influences tardigrade composition (Richardson *et al.*, 2005).

Tardigrade abundance and diversity was also affected by bryophyte host (Chapters 4 and 5), though to a lesser extent than the overall habitat effect. Some hosts had considerably more tardigrades than others, for example, *Pleurozium schreberi* compared with *Chiloscyphus latifolius*. For some tardigrade taxa, hosts appeared to be interchangeable to some degree, while other tardigrades were exclusive to particular hosts. The structural complexity of the host might be partly responsible for any preferences: the more complex structured *Pleurozium* offers a diverse microhabitat whereas the more simply structured *Chiloscyphus* presents fewer options. The role of structural complexity has been demonstrated for invertebrates in plants in other ecosystems (McAbendroth *et al.*, 2005), and for micrororganisms (Kassen and Rainey, 2004). Physical and chemical defences of bryophytes might also act as a selective filter for tardigrades (Swain and Hillis, 1959, Glime, 2006). *Chyloscyphus* the less structurally simple of the hosts and with oil bodies in its leaves had the lowest abundance and diversity, while *Pleurozium* has a reportedly high content of phenolic compounds and yet also hosts many tardigrades (Smith *et al.*, 2001). There are surprisingly few studies of tardigrade associations with hosts, and they have produced mixed conclusions: some found hosts were not important (Bertolani, 1983) while others suggested links between certain taxa and hosts, matching the conclusions on my own studies (Degma, 2003,

Degma and Pecalkova, 2003, Degma, 2006). Clearly, this is an important aspect of tardigrade ecology that deserves much more attention than it has received to date.

Since *Polylepis* forests have been shown to provide a useful model habitat for assessing biogeographical patterns of tardigrades at different scales, it is useful to consider them in more context. This thesis has explored tardigrade patterns at relatively fine scales: for the insights these studies give into the ecology of these animals at such scales, but also to inform the design of wider comparisons in the future at landscape, regional and continental scales. On the basis of these studies, several important recommendations can be made.

Sampling from the forest floor allows direct comparisons with a variety of other habitats lacking the structural complexity of forest trees. Focusing on samples from the forest floor only (ignoring trees) provides the most efficient and easily interpretable way of comparing forest tardigrades with those from habitats, such as bog or grassland, as well as rocky outcrops and riparian zones. Of course, this approach neglects the other parts of the forest structure, where specialist tardigrade species might live. So, for tardigrade inventory studies of whole forests, effective sampling of all parts of the forest structure is recommended.

Sampling should attempt to standardise the size of host samples taken. High variability in abundance and occupancy from sample to sample was characteristic of the datasets presented in this thesis. This pattern is consistent with other studies that have sampled tardigrades quantitatively (Meyer, 2003, GuilSanchez-Moreno *et al.*, 2009, KaczmarekGołdyn *et al.*, 2011), and tardigrades should be assumed to vary in abundance and species richness amongst bryophyte samples (Maucci, 1980, Kathman and Cross, 1991, Degma *et al.*, 2017). This is a major frustration when comparing tardigrade occupancy and abundance data across different sites and studies. The situation is made worse when the sampling units have not been standardised—and this is typical, with most quantitative studies sampling in distinct ways, or failing to describe any standardisation at all. However, it is not clear how standardisation should be carried out. For example, one suggested approach is to sample a set area of host material. But this results in considerable variation between samples of different depths and structural complexity. A set area from a simple hepatic bryophyte, such as *Chiloscyphus*, would yield a small amount of material, compared to the amount collected

from the same area of a *Sphagnum* moss. Therefore, an alternative approach is to standardise by volume of material, rather than area, attempting to include representative material across the structural range of the host (some surface material as well as other material from deep within any cushion structure).

The pilot study included many small samples which ultimately turned out to contain no tardigrades, so larger samples were collected for the studies presented in Chapters 4 and 5. Those studies standardized samples to approximately 4 cm in spherical diameter, representing around 4 cm³ in volume. These later samples almost all contained tardigrades, though they were still highly variable from 0–74 individuals per sample. Other samples collected from *Polylepis* forest—processed but not presented in this thesis—have yielded up to 665 individuals in a single sample, though on average the number is around 50. And this illustrates the other side of the compromise. Although collecting larger samples would help to overcome the problems with occasionally empty samples, it would increase the processing and taxonomic burden considerably.

Replicate samples should be taken from a variety of hosts. The studies in this thesis have shown that some of the rarer tardigrades are restricted to a small number of samples (sometimes just one), often belonging to a single host. The chances of finding these species increases if replicate samples are taken across a variety of hosts within the forest. Where the aim is to compare biogeographical patterns, failing to find taxa could compromise the validity of the conclusions drawn.

In Chapter 5, the analysis indicated that at least 50 samples would be needed to adequately represent tardigrade species richness from that *Polylepis* forest floor. In the same study, distance over fine scales did relate to tardigrade composition. Therefore, it is recommended that at least 50 samples are taken in each forest site, attempting to collect a balanced number from all of the hosts present. On the evidence of this thesis, the samples will remain independent, regardless of the physical distance between them at fine scales. In any case, collecting from a range of hosts and taking replicates should ensure spatial variation between samples.

This research has shown that habitat influences tardigrade abundance, diversity and composition more than host. Different habitats separated by short distances can have very different tardigrade communities. This is an important consideration when comparing sites across broader geographical scales. Comparing different habitats across

distant sites might show differences that results from the habitat scale rather than the scale of interest, completely confounding the results. Thus, sampling strategies for comparing forests at landscape, regional and continental scales should carefully assess the suitability of the forests, rejecting candidate sites which deviate significantly from the required habitat characteristics. Otherwise the data and the conclusions drawn from it might be fundamentally flawed.

In the same vein, wherever possible, the same hosts should be used, though these organisms also have restricted distributions which might limit the extent to which this is possible over long distances (Frahm, 2009).

The model habitat of *Polylepis* woodlands offers considerable advantages for comparisons across a wide range of scales, and the thesis has presented the results of tardigrade studies at fine-scales within the forest itself and comparing it with neighbouring habitats. It has demonstrated the value of considering tardigrade distribution patterns at a variety of scales, and has highlighted the importance of relating host characteristics to tardigrades, and the role of habitat in promoting landscape-level diversity patterns. The thesis also presents a new approach for tardigrade description and identification, using a character matrix, which will facilitate future studies.

Based on the results of the research described, concrete recommendations have been proposed for expanding the research into broader geographical scales: standardising sample volume, replicate sampling across hosts on the forest floor, recognising the importance of habitat-scale effects when selecting study sites, and the development of character matrices for tardigrade genera. It is hoped that this work will lead directly to future studies of tardigrade biogeography at landscape, regional and continental scales, making further contributions to the understanding of these fascinating organisms.

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